Skeletal Muscle Mitochondrial Function in Human Health and Disease

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Summary

Mitochondria are unique cell organelles, also called the powerhouses of our cells, which play a central role in human oxygen metabolism and general energy supply. By creating biologically accessible energy, these organelles sustain the maintenance of metabolic function and homeostasis. Therefore, accurate function of mitochondria is crucial to proper skeletal muscle operations as well as to general human health. Already small alterations or defects of mitochondria may have deleterious effects, which could play an important role in the process of aging and in several neuromuscular and neurodegenerative diseases such as Huntington disease, and in particular in mitochondrial cytopathies. Although the importance of proper mitochondrial function for human health and skeletal muscle functioning has been recognized, a comprehensive understanding of the regulations and the underlying mechanisms especially in relation to aging and the development of various neuromuscular diseases is lacking.

Accordingly, the aim of this comprehensive PhD project was to investigate mitochondrial function and its relation to other skeletal muscle properties in healthy young and old individuals and in patients with disorders that may affect skeletal muscle mitochondria such as mitochondrial myopathy and Huntington disease. Therefore, high-resolution respirometry measurements of permeabilized skeletal muscle fibers were conducted. The application of this method thereby allows the preservation of innate mitochondrial function and represents the highest standard approach for the characterisation of mitochondrial function.

Regarding mitochondrial characteristics, the three independent studies included in this dissertation revealed the following key findings: 1) Maximal fatty acid oxidative capacity was higher in healthy old compared to fitness-level-matched young individuals, 2) mitochondrial respiratory capacity of each complex as well as mitochondrial volume density was lower in patients with mitochondrial myopathy and 3) patients with Huntington disease were characterized by lower complex I respiratory and maximal oxidative phosphorylation capacity compared to healthy controls. Consequently, the present findings contribute to the general understanding of mitochondrial characteristics, potential regulatory mechanisms and adaptations to varying circumstances. Together with previous and future studies these results may potentially lead to the development of effective therapeutic strategies in order to improve mitochondrial function and life quality in aged people as well as in patients with neuromuscular or neurodegenerative diseases.

Zusammenfassung

Mitochondrien sind einzigartige Zellorganellen und spielen eine zentrale Rolle im menschlichen Sauerstoffmetabolismus sowie der generellen Energiebereitstellung. Nicht zuletzt deshalb werden sie auch als Kraftwerke unserer Zellen bezeichnet. Die Bereitstellung biologisch nutzbarer Energie durch diese Organellen dient dabei der Aufrechterhaltung der metabolischen Funktionen unseres Körpers sowie der Homöostase. Gerade deshalb ist ihre Intaktheit sowohl für jegliche Funktionen der Skelettmuskulatur als auch für die allgemeine menschliche Gesundheit entscheidend. Bereits kleine Abweichungen oder Defekte der Mitochondrien bzw. ihrer Funktionsweise können schwerwiegende Folgen nach sich ziehen, welche nicht nur den Alterungsprozess aber auch die Entstehung und den Verlauf von neuromuskulären und neurodegenerativen Krankheiten wie der Huntington-Krankheit und insbesondere auch mitochondriale Zytopathien betreffen. Obwohl die Bedeutung der intakten mitochondrialen Funktion für die menschliche Gesundheit sowie derjenigen der Skelettmuskulatur weitgehend bekannt ist, fehlt bis heute ein ganzheitliches Verständnis für die Regulationen und die zugrundeliegenden Mechanismen, speziell in Bezug auf den Alterungsprozess und die Entstehung neuromuskulärer Krankheiten.

Das Ziel dieses umfassenden PhD-Projektes war es deshalb, die mitochondriale Funktion sowie deren Relation zu anderen Skelettmuskeleigenschaften sowohl in gesunden jungen und älteren Individuen als auch in Patienten mit Krankheiten wie der mitochondrialen Myopathie oder der Huntington-Krankheit, welche die Mitochondrien der Skelettmuskulatur betreffen können, zu untersuchen. Die Untersuchungen wurden dabei mit Hilfe hoch-auflösender respirometrischen Messungen in permeabilisierten Skelettmuskelfasern durchgeführt. Diese Methode erlaubt es, die ursprüngliche Funktionsweise der Mitochondrien zu erhalten und repräsentiert zugleich den höchsten Standard für die Charakterisierung der Mitochondrien.

Bezüglich mitochondrialer Funktion führten die drei unabhängigen Studien der vorliegenden Dissertation zu folgenden Hauptbefunden: 1) Die maximale Fettoxidationsrate der Mitochondrien der Skelettmuskulatur von älteren Individuen war höher als diejenige von jungen in vergleichbarem Fitnesszustand, 2) die mitochondriale respiratorische Kapazität jedes einzelnen Komplexes sowie die mitochondriale Volumendichte waren tiefer in Patienten mit mitochondrialer Myopathie und 3) Huntington-Patienten wiesen im Vergleich zu gesunden Kontrollpersonen tiefere Komplex I Aktivität sowie tiefere maximale oxidative Phosphorylierungs-Kapazität auf. Folglich tragen die vorliegenden Resultate zum generellen

Verständnis mitochondrialer Charakteristika, potentieller regulatorischer Mechanismen sowie Anpassungen an verschiedene Situationen bei. Die hier präsentierten Resultate könnten zusammen mit früheren und zukünftigen Studienergebnissen zur Entwicklung von effektiven therapeutischen Strategien führen, welche die mitochondriale Funktion sowie die Lebensqualität von älteren Menschen sowie Patienten mit neuromuskulären oder neurodegenerativen Krankheiten verbessern könnten.

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1. Introduction

1.1 Metabolism and energy supply

Metabolism describes the entirety of all energy transformations in the human organism. In order to provide the organism with energy, which is critical for all cellular activities and homeostasis, the energy from nutrients needs to be transformed into chemical energy. Appropriate energy supply is also needed for skeletal muscle activity, irrespective of duration and intensity. The human organism obeys the first law of thermodynamics stating that energy is only converted but neither created or destroyed and represents an open thermodynamic system. Accordingly, the chemical energy from nutrients including carbohydrates, fats and proteins is stored as adenosine triphosphate (ATP) and subsequently transferred to energy for physiological functions such as muscular contraction. Thereby, some of the energy is used to conduct physical work but the majority is converted into heat.

The ATP content of skeletal muscle at rest is approximately 25 mmol kg^{-1} dry muscle (Soderlund & Hultman, 1990), which lasts for only a few seconds of contraction. Generally, the ATP stored in the organism at any given time is only sufficient for a few minutes of physiological function. Hence, ATP needs to be constantly resynthesized from ADP that can be realized by interactions of ADP with creatine phosphate, anaerobic glycolysis and aerobic respiration in the cell mitochondria. For their energetically demanding functions as contraction and protein synthesis, skeletal muscle fibers require large amounts of ATP which are particularly provided by mitochondria.

1.2 Mitochondrial structure

Mitochondria are cellular organelles that occupy a substantial proportion of the cytoplasm of eukaryotic cells. Usually, they are described as bean-shaped, stiff, elongated cylinders with a diameter of 0.5 – 1 µm, resembling bacteria (Alberts *et al.*, 2002).

These cell organelles consist of an outer mitochondrial membrane that is permeable to small molecules and ions and separates the mitochondrial intermembrane space from the cytoplasm. In contrast, the inner membrane is highly specialized and impermeable to most small molecules and ions, including protons. The only known transport across the inner membrane from matrix to intermembrane space and vice versa occurs through specific transporters. The inner membrane bears the respiratory chain or electron transport chain

(ETC, complex I-IV) and the ATP synthase (complex V) and encloses the mitochondrial matrix. The ETC itself consists of a series of sequentially acting membrane-embedded multi-enzyme complexes that serve as electron carriers. The inner membrane is also characterized by a series of invaginations, so called cristae, which increase its surface area and project into the matrix. The mitochondrial matrix thereby contains the pyruvate dehydrogenase complex, the enzymes of the tricarboxylic acid cycle (TCA), the fatty acid *β*-oxidation pathway and the pathways of amino acid oxidation. Noteworthy, the matrix also contains the mitochondrial genome.

Mitochondria contain their own genome, the mitochondrial DNA (mtDNA). The human mitochondrial genome is an exclusively maternally inherited double-stranded circular molecule. Each organelle generally contains several identical copies of mtDNA. The mtDNA-encoded polypeptides are all subunits of the enzyme complexes of the ETC. Enzymes of the various metabolic pathways are encoded by nuclear DNA (nDNA). Noteworthy, even the complexes of the ETC are of hybrid origin (Taanman, 1999). Thus, as mitochondria are controlled by mtDNA and nDNA, mutations within either DNA may result in ETC deficiency resulting in different mitochondrial disorders (Tuppen *et al.*, 2010).

Mitochondria are mobile and plastic organelles, changing their shape by fusion and fission (Westermann, 2010). These organelles move in the cytoplasm and are often associated with microtubules, which determine their unique orientation and distribution in different cell types (Alberts *et al.*, 2002). Two morphologically and biochemically distinct populations of skeletal muscle mitochondria can be described: subsarcolemmal (SS) mitochondria and intermyofibrillar (IMF) mitochondria (Müller, 1976; Picard *et al.*, 2013). Morphologically, SS mitochondria rather appear as isolated spherical units and IMF mitochondria are organized as interconnected reticular networks (Figure 1, Kirkwood *et al.*, 1986; Ogata & Yamasaki, 1997; Picard *et al.*, 2013). Subsarcolemmal mitochondria are near the capillary and thought to be involved in providing the energy required for the transport of O_2 from the erythrocyte into the muscle cell (Müller, 1976). In contrast, IMF mitochondria are suggested to provide the energy required for contractile protein interaction and therefore muscular contraction (Müller, 1976). Moreover, mitochondrial shape and configuration are distinctive for each human skeletal muscle fiber type (Ogata & Yamasaki, 1997). Collectively, mitochondria are able to change their configuration in response to cellular metabolism and adapt to varying circumstances, which is critical for maintaining mitochondrial function (Hoppeler & Flück, 2003; Youle & van der Bliek, 2012; Boushel *et al.*, 2014). Thus, mitochondrial morphology and its regulation are distinctively connected with mitochondrial function.

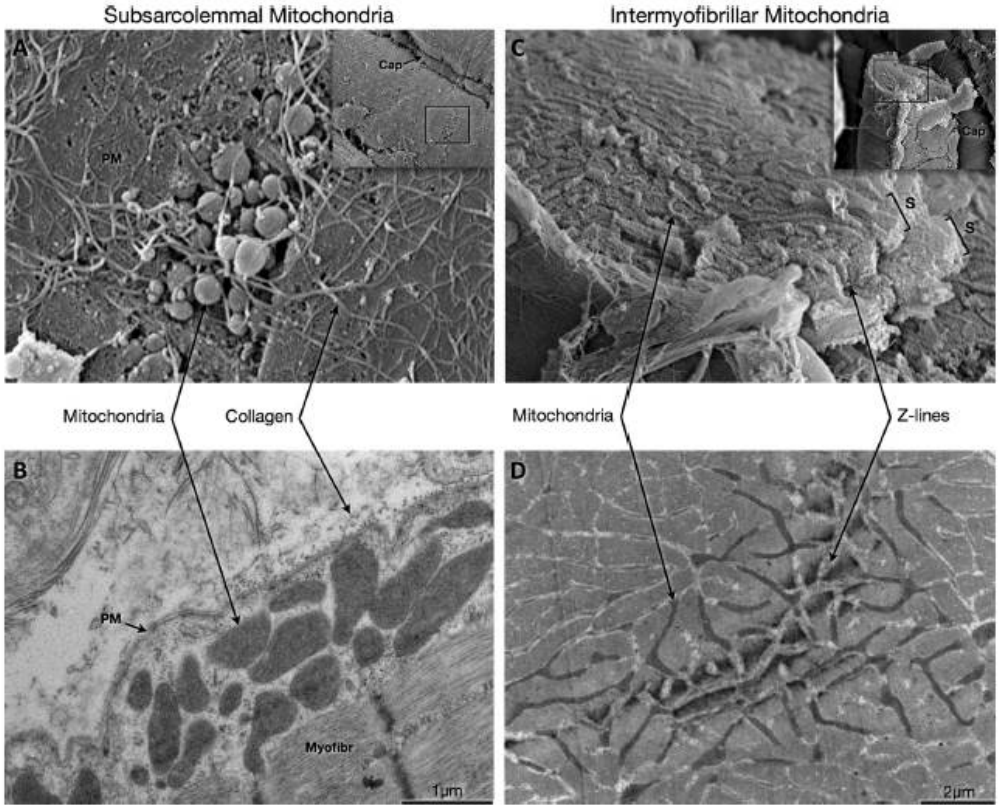

Figure 1. Subsarcolemmal and intermyofibrillar mitochondrial morphology. Electron microscopic image of a freeze-fractured intact mouse soleus muscle. Cap, capillary; Myofibr, myofibrils; PM, plasma membrane; S, sarcomeres. (modified from Picard *et al.*, 2013)

1.3 Mitochondria and their critical role in energy supply

The primary production of ATP is achieved by cellular respiration in the mitochondria. Cellular respiration describes the molecular process by which cells consume O_2 and produce CO_2, occurring in three major stages (Nelson & Cox, 2008). Firstly, glucose, fatty acids and some amino acids are oxidized to acetyl-coenzyme A (acetyl-CoA). Secondly, acetyl-CoA enters the TCA (Figure 2), whereby acetyl-CoA is oxidized to CO_2 and the released energy is conserved in the form of NADH and $FADH_2$. Thirdly, NADH and $FADH_2$ are oxidized. Thereby, the electrons are transferred to O_2 via the ETC. The amount of energy released is conserved as ATP by a process named oxidative phosphorylation (Nelson & Cox, 2008).

Oxidative phosphorylation is regulated by cellular energy demands and described by three main processes (Figure 3): 1) flow of electrons through a chain of membrane-bound carriers, 2) coupling of exergonic electron flow out of the matrix into the intermembrane space to endergonic transport of protons across the inner membrane, thereby conserving the energy

as a transmembrane electrochemical potential, and 3) the transmembrane flow of protons down their concentration gradient that provides the energy for the synthesis of ATP by ATP synthase, a membrane-bound protein complex that couples proton flow to phosphorylation of ADP (Mitchell, 1961; Nelson & Cox, 2008).

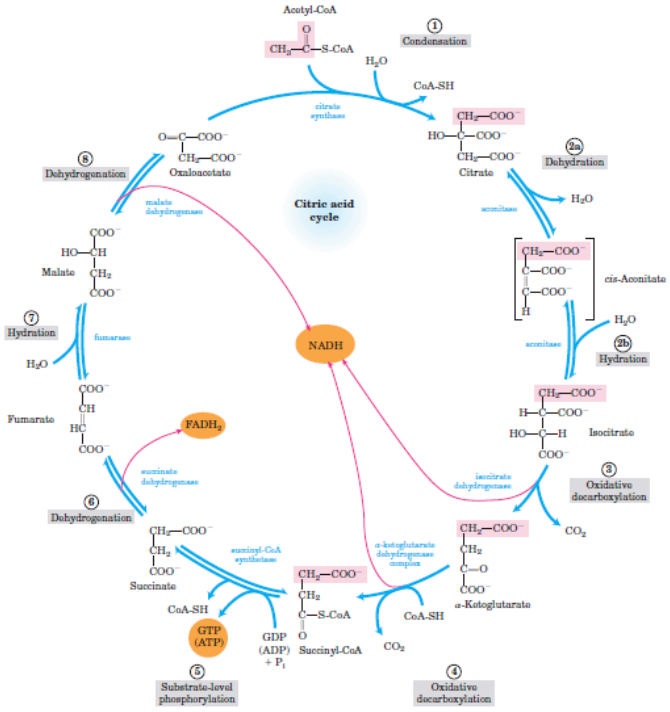

Figure 2. The tricarboxylic acid cycle (TCA). Acetyl-CoA is oxidized to CO_2 and the released energy is conserved in NADH and $FADH_2$. The TCA itself produces one ATP (or GTP) at each turn. (modified from Nelson & Cox, 2008)

The pumping of protons across the inner membrane results in a pH gradient with a higher pH in the matrix than in the intermembrane space and a voltage gradient (the membrane potential), with a negatively charged matrix and a positively charged intermembrane space. Together the gradients build the so called electrochemical proton gradient, which exerts a proton motive force and drives the ATP synthesis in the process of oxidative phosphorylation (Alberts *et al.*, 2002).

Oxidative phosphorylation starts with the entrance of electrons into the ETC. Electron input thereby occurs from the following four sources (Figure 4): 1) NADH, 2) TCA specific $FADH_2$ (succinate), 3) electrons from metabolized fatty-acids through the electron-transferring flavoprotein (ETF), and 4) electrons from glycerol 3-phosphate. All electrons are transferred to ubiquinone (Q).

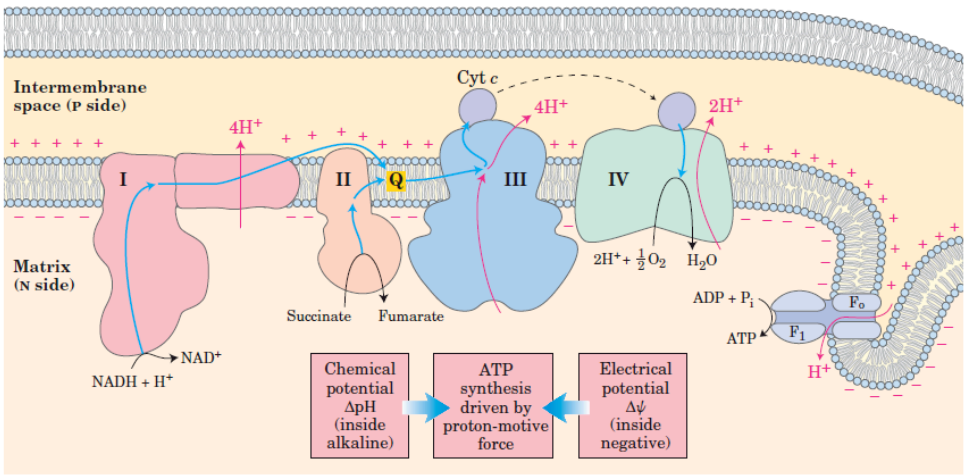

Figure 3. The respiratory chain. Simple representation of the chemiosmotic theory applied to mitochondria. The transmembrane differences in protein concentration are the reservoir for the energy that is extracted from biological oxidation reactions. (modified from Nelson & Cox, 2008)

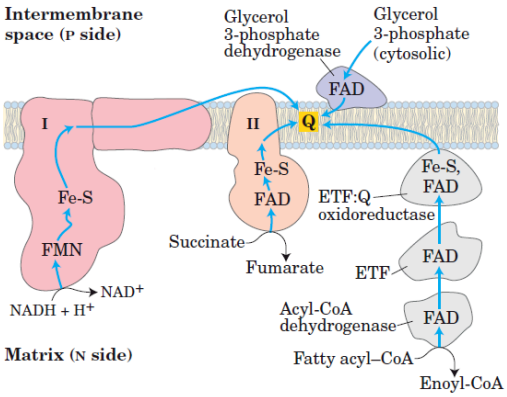

Figure 4. Electron input into the mitochondrial respiratory chain from NADH, succinate, fatty acyl-CoA and glycerol 3-phosphate to ubiquinone (Q). Electrons from NADH pass through complex I and electrons from succinate through complex II to Q. Electrons from *β*-oxidation are transferred to electron-transferring flavoprotein (ETF) that passes them to Q. (modified from Nelson & Cox, 2008)

In Complex I (CI) electrons are transferred from NADH to Q whereby simultaneously protons are pumped from the matrix into the intermembrane space. Complex II (CII) is the only membrane-bound enzyme of the TCA and thereby allows the electron transfer from succinate via FAD to Q. Other substrates pass electrons into the ETC at the level of Q either via FAD and ETF or by glycerol 3-phosphate dehydrogenase (Figure 4). Reduced Q (QH_2) is subsequently reoxidized by Complex III (CIII). Accordingly, CIII couples the transport of electrons from QH_2 to cytochrome C (CytC), a soluble protein of the intermembrane space.

As a result, CytC donates the electron to complex IV (CIV). Finally, CIV, better known as cytochrome c oxidase (COX), carries electrons from CytC to O_2 and reduces O_2 to H_2O. In total, for each pair of electrons transferred to O_2, four protons are pumped out by CI, four by CIII and two by CIV (Figure 3), resulting in the previously described proton motive force. This proton motive force in turn drives the synthesis of ATP from ADP and P_i by complex V (CV), also called ATP synthase. Several steps in the path of oxidative phosphorylation have the potential to produce reactive oxygen species (ROS) that can damage cells. When the rate of electron entry into the ETC and the rate of electron transfer through the ETC are mismatched, superoxide radical production increases at CI and CIII. These potentially harmful ROS can to a certain amount be inactivated by a set of protective enzymes as superoxide dismutase (Nelson & Cox, 2008).

Fundamentally, skeletal muscle fibers are densely populated with these organelles and are able to substantially increase their ATP production during maximal contraction (Bangsbo *et al.*, 2001). Thus, proper functioning mitochondria represent an essential requirement for sustained ATP production in order to maintain physiological homeostasis and skeletal muscle work.

1.4 Mitochondrial function and its critical role in human physiology

In addition to energy supply, mitochondria have several other functions in specific tissues and under distinct circumstances that are also crucial for the maintenance of homeostasis and human health. Amongst others, mitochondria are the sites of steroid hormone synthesis (Miller, 2013), they control cytosolic calcium concentration (Pozzan *et al.*, 2000) and regulate apoptotic cell death (Newmeyer & Ferguson-Miller, 2003). The primary function of mitochondria, however, is the production of ATP by oxidative phosphorylation, as described above. When referring to mitochondrial function throughout this dissertation, this specifically refers to the bioenergetic characteristics in human skeletal muscle mitochondria.

Mitochondria are dynamic subcellular structures and continuously adapt to their actual environment by alterations of size and number (Hoppeler & Flück, 2003; Youle & van der Bliek, 2012; Boushel *et al.*, 2014). The important role of this organelle in metabolic homeostasis represents both a blessing and a curse. On the one hand, metabolic stress like exercise can positively influence mitochondrial function (Jacobs *et al.*, 2013; Boushel *et al.*, 2014) but on the other hand, already small alterations or little damage of those organelles can have severe consequences on human health (Zeviani & Di Donato, 2004; Tuppen *et al.*,

2010). Nowadays, the importance of proper mitochondrial function in relation to muscle physiology is undisputed. Nonetheless, several skeletal muscle mitochondrial characteristics especially their role in the process of aging and different diseases are not completely understood. Revealing the mechanisms regulating mitochondrial characteristics and their relations to other skeletal muscle properties could be crucial to further increase the present understanding of mitochondrial and muscle physiology and pathophysiology, respectively. Accordingly, investigating mitochondrial function and its adaptation to distinct stimuli or changing circumstances, respectively, is of great importance in the understanding of the process of aging and several diseases affecting the muscle and nervous system such as mitochondrial myopathy (MM) and Huntington disease (HD).

The investigation of skeletal muscle mitochondrial function and its relations to human health including the process of aging and disease require the administration of sensitive and reliable functional measures. Classic studies have measured function from maximal respiratory capacity in isolated mitochondria, which provides a mechanistic insight in mitochondrial characteristics. However, the state-of-the-art in this field is a twin-chamber instrument called Oroboros Oxygraph-2k (Oroboros Instruments, Innsbruck, Austria), which only needs small tissue amounts and is highly sensitive (Gnaiger, 2009; Lanza & Nair, 2010). Moreover, this approach allows the assessment of respiratory capacity at multiple levels of the respiratory chain, serial measurements in the same tissue sample and the determination of oxidative phosphorylation efficiency (Gnaiger, 2001). All studies of this dissertation applied in vitro high-resolution respirometric assessments of skeletal muscle mitochondrial function. Since mitochondrial isolation procedures may disrupt the functional structural network and alter innate mitochondrial characteristics (Picard *et al.*, 2011), measures were conducted in permeabilized myofibers. Hence, high-resolution respirometry measurements in permeabilized fibers allow the preservation of innate mitochondrial function and represent the highest standard approach for the investigation of mitochondrial characteristics. In order to provide a qualitative index of mitochondrial function, polarographic measurements of O_2 consumption are frequently normalized to mitochondrial content. Accordingly, accurate determination of mitochondrial content is crucial to any measure of intrinsic mitochondrial function. Two-dimensional imaging using transmission electron microscopy (TEM) is regarded as the gold standard to assess mitochondrial content. However, since TEM measurements are time consuming and are not available for many laboratories, biomarkers have often been applied as a surrogate for mitochondrial content. These biomarkers include mitochondrial enzyme activities such as citrate synthase (CS) or COX and have become widely accepted, despite varying correlations with TEM-assessed mitochondrial content

(Larsen *et al.*, 2012). In the present studies, different methods for the assessment of mitochondrial content have been applied, including the gold standard of TEM as well as two different biomarkers.

The following subsections briefly elucidate the specific reasons for the conductance of the three studies included in the present dissertation. The detailed background information, methods, results and conclusions are presented in the manuscript section. The manuscripts are to date in second revision, ready to submit or in preparation for submission.

1.4.1 Aging, efficiency and skeletal muscle mitochondrial function

Exercise efficiency (EE) describes the effectiveness of the human body in converting energy from nutrients into external work and is therefore defined as the ratio of work performed to the amount of oxygen consumed (Gaesser & Brooks, 1975). In the present literature there is still debate about whether and in which direction EE is altered with aging (Ortega, 2013; Venturelli & Richardson, 2013). A possible explanation for the ongoing discussion and for the discrepancies between previous findings could be varying fitness level of the study participants. In general, old individuals are suggested to be less trained than young individuals (Russ & Kent-Braun, 2004; Amati *et al.*, 2008; Gram *et al.*, 2014), which could introduce a bias as EE has been demonstrated to differ between trained and untrained individuals (Hopker *et al.*, 2013) and to be improved with exercise training (Woo *et al.*, 2006; Montero & Lundby, 2015). Additionally, peak O_2 uptake ($\dot{V}O_{2peak}$) declines at a rate of approximately 4.0 mL kg^{-1} min^{-1} (8 %) per decade due to differences in maximum heart rate (Wilson & Tanaka, 2000). The first aim of this study was to determine EE in fitness-level-matched young and old individuals to test the hypothesis that EE is higher in the old population. In order to match the participants by similar fitness level, $\dot{V}O_{2peak}$ was adjusted for age in advance (Wilson & Tanaka, 2000). The second aim of this study was to elucidate the potential sources for the expected differences with age. We hypothesized that the ratio of slow-twitch (ST) to fast-twitch (FT) fiber is higher in old individuals. Moreover, we assumed that mitochondrial function is preserved with age when controlling for fitness level, however, mitochondrial content is higher according to higher ST fiber proportion, which could explain a higher EE with advanced age.

1.4.2 Mitochondrial myopathy (MM) and skeletal muscle mitochondrial function

Mitochondrial cytopathies (MC) are a group of clinically heterogeneous diseases, commonly defined by a lack of cellular energy (Tuppen *et al.*, 2010). MC are caused by mutations of mtDNA and/or nDNA, which encode for mitochondrial proteins (Wallace, 1999) and may therefore lead to defects in either one or several mitochondrial metabolic pathways including the respiratory chain and oxidative phosphorylation. Besides central-nervous system manifestations, MC also include neuromuscular manifestations such as myopathy and peripheral neuropathy (Taylor & Turnbull, 2005). Specifically, the affection of skeletal muscle is defined as mitochondrial myopathy (MM) and represents a common manifestation of MC, either as isolated MM or in combination with other symptoms of MC (Di Mauro, 2004). Despite constant progress in understanding the biochemistry and genetics of MM, pathophysiological mechanisms have remained elusive and proven cures as well as distinct diagnostic criteria are lacking (Di Mauro, 2010; Hassani *et al.*, 2010). Defects in the muscle oxidative phosphorylation cascade could result in exercise intolerance and muscle fatigue, often reported symptoms, leading to substantially impaired quality of life and a sedentary lifestyle (Taivassalo & Haller, 2004). The latter could in turn further decrease the level of functional mitochondria. Therefore, the purpose of this study was to determine mitochondrial function in patients with MM in comparison to healthy age- and gender-matched individuals. In order to being able to characterize mitochondrial function and potential pathomechanisms, we aimed to assess the respiratory capacity of each single complex.

1.4.3 Huntington disease (HD) and skeletal muscle mitochondrial function

Huntington disease (HD) is an autosomal-dominant, progressive neurodegenerative disorder with various symptoms including cognitive, behavioural and motor dysfunction (Martin & Gusella, 1986; Walker, 2007). HD is caused by mutated huntingtin, which results from an expanded CAG trinucleotide repeat leading to a poly-glutamine strand of variable length (The Huntington's Disease Collaborative Research Group, 1993). To date, it is suggested that this poly-glutamine stretch confers a toxic gain of function. However, precise pathophysiological mechanisms are not fully understood (Walker, 2007). Accordingly, therapeutic approaches that slow the progressive dysfunction are currently unavailable. Considering the involvement of the motor system, thorough analysis of skeletal muscle properties could offer promising insights into potential pathomechanisms in peripheral tissue. First studies indicate a deficit of in vivo mitochondrial oxidative metabolism, supporting the role of mitochondrial dysfunction

as a factor in the pathogenesis of HD (Lodi *et al.*, 2000; Saft *et al.*, 2005). Therefore, the aim of this study was to investigate potential alterations in vitro mitochondrial respiratory capacity of each complex and in skeletal muscle morphology of patients with HD. We hypothesized that HD also affects and manifests in peripheral tissue, in addition to the already known central-nervous system manifestations. These investigations could thereby offer new insights into the pathogenic mechanisms which could in the future lead to new therapeutic approaches.

In conclusion, the main purpose of the present PhD project was to generate a comprehensive assessment of mitochondrial function by investigating mitochondrial function in healthy young and old individuals and in patients with diseases including mitochondrial affections such as MM and HD. Another aim of this study was to investigate the relations of mitochondrial function to other muscle physiological parameters as exercise performance, exercise efficiency and muscle phenotype. A potential implication could be the enhancement of diagnostic and classification measures of MM, as these presently require a multifaceted approach of numerous methods (Tarnopolsky & Raha, 2005). Last but not least, effective treatment approaches for MM and HD are currently lacking (Di Mauro, 2010; Hassani *et al.*, 2010), thus novel therapeutic treatments are urgently needed. Therefore, the present aspired contribution to the understanding of the molecular basis of these conditions may support the development of novel therapeutic strategies to improve mitochondrial function and to delay the onset of (age-related) diseases in the future.

2. Manuscripts

2.1 Exercise efficiency is higher in old compared to young men at similar fitness level

in revision

2.2 Patients with mitochondrial myopathy exhibit diminished mitochondrial quantity and quality and a shift in skeletal muscle phenotype

in submission

2.3 Mitochondrial function in skeletal muscle of Huntington disease patients

in preparation for submission

Exercise efficiency is higher in old compared to young men at similar fitness level

in revision

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Abstract

Purpose Whether exercise efficiency (EE) becomes elevated with age is debated and the underlying mechanisms remain disputed. Thus, the aim of this study was to determine if EE is higher in old humans and whether these potential differences may be attributed to skeletal muscle properties. **Methods** EE was assessed in 11 young (27 ± 4 yr) and 12 old (66 ± 4 yr) individuals of matched fitness levels. Skeletal muscle biopsies were obtained from the *m. vastus lateralis* for the assessment of fiber type distribution, citrate synthase activity and mitochondrial function. Leg mass was determined by dual-energy X-ray absorptiometry. **Results** EE was higher (P < 0.01) in old (16.7 ± 1.5 %) than young (14.0 ± 2.4 %) individuals, whereas leg mass was similar (P = 0.457). Also percentage slow- and fast-twitch fiber distribution did not differ (61.3 ± 11.7 vs. 53.4 ± 10.9 % and 38.7 ± 11.7 vs. 46.6 ± 10.9 %, P = 0.161). Similarly, citrate synthase activity, maximal mitochondrial oxidative phosphorylation capacity and measures of mitochondrial efficiency did not differ. Submaximal respiratory exchange ratio was however lower (P < 0.001) in the old (0.80 ± 0.06 and 0.95 ± 0.07, respectively), which coincided with higher (P < 0.001) maximal fatty acid oxidative capacity (36.2 ± 9.9 vs. 20.5 ± 3.7 pmol O_2 mg^{-1} s^{-1}) and that revealed a positive correlation with EE (P < 0.01). **Conclusions** EE is higher in older individuals when matched for fitness level and is correlated with fatty acid oxidative capacity.

Keywords

Aging; bioenergetics; mitochondrial function; whole body economy

Abbreviations

AU, arbitrary unit; BMR, basal metabolic rate; BSA, bovine serum albumin; COX, respiratory capacity of complex IV; CS, citrate synthase; DE, delta efficiency; DXA, dual-energy X-ray absorptiometry; E, electron transport system capacity; EC, exercise economy; EE, exercise efficiency; ETF_{EFF}, coupling efficiency of fat respiration; ETS, electron transport system; FCCP, carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone; FT fibers, fast-twitch fibers; GE, gross efficiency; LCR, leak control ratio; L_N, leak respiration without adenylates; L_{Omy}, oligomycin-induced leak respiration; mATPase, myofibrillar adenosinetriphosphatase; MHC, myosin heavy-chain; MIT_{EFF}, mitochondrial coupling efficiency; OLD, old participants; P, oxidative phosphorylation capacity; P_{CI}, respiratory capacity of complex I; P_{CII}, respiratory capacity of complex II; P_{ETF}, fatty acid oxidative capacity; RER, respiratory exchange ratio; ROS, residual oxygen species; ROX, residual oxygen consumption; SOD2, mitochondrial superoxide dismutase 2; ST fibers, slow-twitch fibers; TBST, tris-buffered saline and 0.1 % Tween-20; $\dot{V}O_{2peak}$, peak oxygen uptake; ww, wet weight; YNG, young participants.

Introduction

Exercise efficiency (EE) is a measure of the whole body's effectiveness in converting energy from nutrients into external work, i.e., the ratio of power output to the total metabolic energy cost (Gaesser and Brooks 1975). Whether EE is altered with aging is a matter of debate. Several studies have demonstrated an age-dependent increase in EE (Tevald et al. 2010; Venturelli and Richardson 2013; Venturelli et al. 2013), whereas others have not (Hopker et al. 2013; Sacchetti et al. 2010; Woo et al. 2006). One plausible explanation for the discrepancies between study outcomes could be fitness level since, generally, old individuals are suggested to be less trained than young persons (Amati et al. 2008; Gram et al. 2014; Russ and Kent-Braun 2004). This may introduce a bias as EE has been demonstrated to differ between trained and untrained individuals (Hopker et al. 2013) and may furthermore be improved with exercise training (Montero and Lundby 2015; Woo et al. 2006). Thus far, only Woo et al. (2006) and Hopker et al. (2013) included trained and untrained individuals of different age groups (20-33 and 50-77 yr); however, they did not match the groups according to peak oxygen uptake ($\dot{V}O_{2peak}$). Since $\dot{V}O_{2peak}$ declines at a rate of approximately 4.0 mL kg^{-1} min^{-1} (8 %) per decade (Wilson and Tanaka 2000), this needs to be accounted for if young and old individuals are to be matched with regards to fitness level. Accordingly, one first aim of the present study was to assess EE in fitness-level-matched young (< 30 yr) and old (> 60 yr) individuals to test the hypothesis that EE is higher in the old population despite controlling for fitness level.

Although individual variations in EE, irrespective of age, have been known to exist for decades (Gaesser and Brooks 1975), the underlying mechanisms remain elusive. Variations in EE are likely of multifactorial origin, including anatomical (Bramble and Lieberman 2004), biomechanical (Kyrolainen et al. 2000) and biochemical parameters (Mogensen et al. 2006) as well as muscle morphology (Coyle et al. 1992; Mogensen et al. 2006). Factors related to skeletal muscle morphology such as a high percentage of slow twitch (ST) fiber distribution have been correlated with a superior EE in some studies (Coyle et al. 1992; Mogensen et al. 2006), but refuted in others (Hopker et al. 2013). With respect to age, preserved fiber type distribution or even a higher proportion of ST fibers and an increased area of ST fibers secondary to fast twitch (FT) fiber atrophy have been reported (Hopker et al. 2013). These findings are consistent with the observed denervation being a primary cause of myofiber atrophy at the myocyte level in the aging muscle (Rowan et al. 2012). A further aim of the present study was to determine the association between EE and skeletal muscle fiber type

distribution in old and young individuals to test the hypothesis that ST fiber distribution is higher in old individuals at similar fitness level and thus may explain the expected higher EE.

Moreover, the reported association between skeletal muscle ST fibers and EE could be related to the often greater mitochondrial content in ST fibers compared to FT fibers (Jackman and Willis 1996). Thus, we also aimed to correlate citrate synthase (CS) activity, which has been suggested a suitable marker for mitochondrial content across individuals (Larsen et al. 2012b), to EE. Additionally, exercise has been reported to revert potential decreases in mitochondrial content with age (Broskey et al. 2013). We therefore hypothesized that a higher EE with age is associated with a higher distribution of ST fibers and, accordingly, higher mitochondrial content in the old compared to the young population when considering similar levels of fitness. Furthermore, Mogensen et al. (2006) assessed skeletal muscle mitochondrial efficiency in an attempt to explain differences in EE observed across young individuals, but did not find any correlation between these measures. Skeletal muscle mitochondrial function was however determined in isolated mitochondria and expressed per CS activity (Mogensen et al. 2006), and regrettably mitochondrial isolation disrupts the complex structural network and may thereby alter innate mitochondrial characteristics (Picard et al. 2010). Moreover, functional impairment of mitochondria with aging is exaggerated in isolated compared to permeabilized muscle fibers (Picard et al. 2010). In contrast to the isolation procedure, permeabilization of myofiber bundles preserves mitochondrial morphology and integrity and hence allows to investigate the intact mitochondrial network. Unaltered mitochondrial function with age has been verified in permeabilized fibers (Gram et al. 2014; Larsen et al. 2012a). However, in one of these studies (Larsen et al. 2012a), the old and young individuals had similar $\dot{V}O_{2peak}$, wherefore it cannot be definitely ruled out if the observed similar mitochondrial function was the result of a relatively better trained old population (Jacobs et al. 2013). Thus, another aim of this study was to test whether mitochondrial function assessed in permeabilized muscle fibers is comparable in old and young individuals at the same relative level of fitness. According to the findings of Gram et al. (2014), we hypothesized that mitochondrial function is preserved with age when controlling for fitness level and that a higher content of similarly functioning mitochondria leads to higher EE in old compared to young individuals.

In summary, the purpose of the present study was to investigate whether EE is higher in old compared to young fitness-level-matched men and simultaneously to elucidate the potential source for the difference.

Methods

Ethical approval

The present experimental protocols were approved by the Ethical Committee of the ETH Zurich (ETH, EK 2011-N-24) and the Cantonal Ethics Committee of Zurich (2013-0114) and were conducted in accordance with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. Participants were fully informed about the purposes, benefits and risks associated with this study and gave their written informed consent prior to the initiation of the experiments.

Participants

A total of 23 male individuals, 11 young (27 ± 4 yr, YNG) and 12 old (66 ± 4 yr, OLD) voluntarily participated in this study. Participants' characteristics are presented in Table 1. All participants were recreationally active; they were neither sedentary nor highly trained. Apart from three OLD individuals with medically-controlled hypertension, all other participants were healthy and none had coronary heart disease, peripheral vascular disease, or clinically significant hyperlipidemia. When applicable, study participants answered specific health questions and provided a medical certificate of health. YNG and OLD were recruited for two separate exercise intervention studies, wherefrom one subset of data has already been published previously (Jacobs et al. 2013). All data published here have been separately analyzed for this study.

Experimental design

The experiments consisted of a skeletal muscle biopsy obtained from the *m. vastus lateralis*, a scan for body composition and an incremental exercise test to assess $\dot{V}O_{2peak}$. All experiments were conducted on separate days.

Body composition measurement

A densitometer (Lunar iDXA^{TM}, GE Healthcare, Madison, WI, USA) was used for the determination of total and lean soft tissue mass of the leg (leg_{total}, leg_{lean}) by performing dual-energy X-ray absorptiometry (DXA) measurements.

Exercise testing

Each participant conducted an incremental exercise test on an electrically braked cycle ergometer until voluntary exhaustion (YNG: Monark E839, Varberg, Sweden; OLD: Ergoselect 200K, Ergoline, Bitz, Germany) to determine $\dot{V}O_{2peak}$ and EE. Pulmonary gas exchange and ventilation were continuously recorded using an online gas collection system (Innocor™ M400, Innovision, Odense, Denmark), where O_2 and CO_2 concentration were continuously measured and monitored as breath-by-breath values. The gas analysers and the flowmeter of the applied spirometer were calibrated prior to each test according to the manufacturer's instructions. Protocols of the incremental tests differed between YNG and OLD, as the OLD were expected not to reach as high power outputs as the YNG. Therefore, the OLD started exercise at 0 W and power was increased by 20 W every 120 s until volitional fatigue. The YNG began with three consecutive submaximal 5-min power stages, starting at 50 W which was then increased to 100 and 150 W. Thereafter, workoad was increased by 30 W every 90 s until voluntary exhaustion. Pedal cadences were freely chosen by the participants but had to remain constant throughout the test and lay between 60 and 90 rpm in YNG and OLD. $\dot{V}O_{2peak}$ was determined as the highest mean over a 10-s period. With the intent to reduce the confounding factor of fitness level, $\dot{V}O_{2peak}$ of the OLD were adjusted for age by adding 4 mL kg^{-1} min^{-1} per decade (Wilson and Tanaka 2000) and subsequently matched to the YNG. Consequently, fitness levels were determined as $\dot{V}O_{2peak}$ which were adjusted for age according to the decreases in maximal heart rate with age (Wilson and Tanaka 2000).

Exercise efficiency (EE)

Calculations of EE from the incremental exercise tests were based on indirect calorimetry and the assumption that energy requirements were met by respiration. Gross efficiency (GE), delta efficiency (DE) and exercise economy (EC) were determined from $\dot{V}O_2$ of submaximal power values. GE was calculated using absolute (100 W, GE_{100}) and relative (GE_{rel}) exercise intensities. For the calculation of GE_{rel} and DE, $\dot{V}O_2$ was averaged over the last 30 s at approximately 50 %, 60 % and 70 % $\dot{V}O_{2peak}$. The according power stages (YNG vs. OLD: 50 %: 77 ± 34 vs. 63 ± 12 W; 60 %: 116 ± 29 vs. 88 ± 18 W; 70 %: 135 ± 38 vs. 107 ± 21 W) and $\dot{V}CO_2$ (mean over last 30 s) were extracted from the incremental exercise test (on the basis of Coyle et al. 1992). GE_{100} and GE_{rel} were calculated as the ratio of work accomplished to energy expended (Coyle et al. 1992; Gaesser and Brooks 1975). GE_{rel} was finally determined as the mean of all three submaximal power stages. To eliminate the influence of the basal metabolic rate (BMR) on energy expenditure, DE was calculated as the

ratio of change in work accomplished to change in energy expenditure (Coyle et al. 1992). For each subject DE was estimated from the reciprocal of the slope of the linear trend line of the relationship between energy expenditure and work accomplished (Coyle et al. 1992). To determine EC, $\dot{V}O_2$ was determined as the mean from 60 - 120 s at 100 W to obtain a steady-state value. EC was calculated for each subject by dividing power (100 W) by the according $\dot{V}O_2$ (Moseley and Jeukendrup 2001). The respiratory exchange ratios (RER) for submaximal power values were also calculated as the mean from 60 - 120 s at 100 W (RER_{100}) and the mean from the ventilation and power stages according to 50 %, 60 % and 70 % $\dot{V}O_{2peak}$ (RER_{rel}).

Skeletal muscle sampling

Skeletal muscle biopsies were obtained under standardized conditions from the *m. vastus lateralis* under local anesthesia (1 % lidocaine) of the skin and superficial muscle fascia, using the Bergström technique with a needle modified for suction. The biopsy was immediately dissected macroscopically free of fat and connective tissue and divided into sections for measurements of mitochondrial respiration, CS activity and oxidative stress as well as muscular fiber distribution. The part of the biopsy for the determination of mitochondrial respiratory capacity was immediately placed in ice-cold biopsy preservation solution. The samples for measurements of CS activity and oxidative stress were frozen in liquid nitrogen and stored at −80 °C until analyzed. Tissue for the determination of muscle fiber type distribution was instantly mounted in an embedding medium (Tissue-Tek®, Sakura, Zoeterwoude, The Netherlands), snap frozen in isopentane cooled to −160 °C with liquid nitrogen, and subsequently stored at −80 °C until use.

Skeletal muscle fiber typing

Consecutive 8 µm sections were cut on a microtome at −25 °C and mounted on glass cover slides for further histochemical analyses. The serial cryocut-cross-sections were stained using the myofibrillar adenosinetriphosphatase (mATPase) method after acid (pH 4.6) and alkali (pH 10.5) preincubation according to Item et al. (2011). For all analyses, only fibers fully encircled by adjacent fibers were evaluated using Adobe Photoshop Pro CS6 (Adobe Systems Incorporated, San Jose, CA, USA) and 812 ± 364 muscle fibers in YNG and 227 ± 170 in OLD, respectively, were classified into ST and FT fibers. Fiber cross-sectional area (CSA) was determined by encircling the boundaries of the muscle cells of at least 50 fibers per fiber type. Only fibers with a cicularity higher than 0.7 were considered for analysis (perfect circle = 1.0).

Mitochondrial respiration measurement

Samples were prepared as described in detail previously (Jacobs et al. 2012). In short, after mechanical fiber separation, chemical permeabilization in biopsy preservation solution and washing in mitochondrial respiration medium 05, respectively, muscle bundles were blotted dry and measured for wet weight (ww) in a balance-controlled scale (XS205 DualRange Analytical Balance, Mettler-Toledo AG, Greifensee, Switzerland). Respiration measurements were then performed in mitochondrial respiration medium 06. Oxygen consumption of the individual muscle tissue was measured at 37 °C using the high-resolution Oxygraph-2k (Oroboros, Innsbruck, Austria) with the titration of each substrate, uncoupler and inhibitor in series. Standardized instrumental and chemical calibrations were performed as recommended by the manufacturer and described previously (Jacobs et al. 2012). Oxygen flux was automatically calculated by the software, accounting for nonlinear changes in the negative time derivative of the oxygen concentration signal (DatLab, Oroboros, Innsbruck, Austria). All experiments were accomplished as duplicates in a hyperoxygenated environment to prevent any potential oxygen diffusion limitation. Oxygen concentration within the chambers ranged between 200 and 450 nmol mL^{-1}.

Respiratory titration protocol

The applied protocol was specific to the analysis of individual aspects of respiratory capacity and coupling control efficiency during several substrate states induced via separate titrations. All titrations were added in series as presented, whereby the concentrations of substrates, uncouplers and inhibitors used were based on prior experiments (Jacobs et al. 2012). This titration protocol was modified from previous protocols where they are described in detail (Jacobs et al. 2012). Leak respiration in absence of adenylates (L_N) was induced with the addition of octanoyl carnitine (0.2 mM) and malate (2 mM). The L_N state represents the resting oxygen consumption of an unaltered and intact electron transport system (ETS) free of adenylates. Maximal electron flow through electron-transferring flavoprotein and maximal fatty acid oxidative capacity (P_{ETF}) was determined following the addition of ADP (5 mM). Submaximal state 3 respiratory capacity representative of electron capacity through complex I (P_{CI}) was induced following the additions of pyruvate (5 mM) and glutamate (10 mM). Maximal state 3 respiration, oxidative phosphorylation capacity (P), was then induced with the addition of succinate (10 mM). P represents respiration that is resultant to saturating concentrations of ADP and substrate supply both for complex I and II. As an internal control for the integrity of the mitochondrial preparation, the mitochondrial outer membrane was assessed with the addition of cytochrome C (10 µM). Titration of oligomycin (1 µM) inhibited

ATP synthase and lead to oligomycin-induced leak respiration (L_{Omy}). L_{Omy} represents the corresponding leak state to P. In L_{Omy} the chemiosmotic gradient is at maximum because of maximal substrate supply and inhibition of ATP synthase. Moreover, oxygen flux is at minimum and is representative of proton leak, slip, cation cycling and overall dyscoupling. Phosphorylative restraint of electron transport was assessed by uncoupling ATP synthase from the electron transport chain with the step-wise titration (4 x 0.5 µM) of the proton ionophore carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone (FCCP), reaching ETS capacity (E). Rotenone (0.5 µM) was added to inhibit CI and to thereby assess electron flow specific to complex II (P_{CII}). P_{CII} is not influenced by the preceding addition of FCCP, which was verified with separate protocols in our laboratory. The addition of antimycin A (2.5 µM) that inhibits complex III allows the determination and correction of residual oxygen consumption (ROX), which is indicative of non-mitochondrial oxygen consumption in the chamber. Respiration measurements were terminated by simultaneous titration of ascorbate and TMPD to assess complex IV (COX) activity. TMPD and ascorbate are redox substrates that donate electrons directly to COX. Correction of O_2 flux for the side reaction of auto-oxidation was conducted by chemical calibration experiments prior to the measurements. Finally, mitochondrial leak control ratios (LCR) were analyzed as well. LCR are produced between two respiratory states, a leak state (low respiration) to a higher respiratory state. The corresponding states are paired by an identical substrate supply. The reference state is defined by the leak state. LCR allow the description of mitochondrial coupling efficiency, with a theoretical minimum of 0.0, indicating a fully coupled system, to a value of 1.0, representing a fully uncoupled system (Gnaiger 2009; Jacobs et al. 2012). Accordingly, LCR of L_{Omy} to E is indicative of coupling efficiency across the entire respiratory chain and representative of mitochondrial coupling efficiency (Mit_{EFF}). Similarly, LCR of L_N to P_{ETF} represents the coupling efficiency of fat respiration (ETF_{EFF}).

Homogenization

Muscle samples were weighed (XS205 DualRange Analytical Balance; Mettler-Toledo AG, Greifensee, Switzerland) and put into cryotubes with ceramic beads (hard tissue homogenizing CK28, 2 mL, Precellys, Bertin Technologies, Montigny-le-Bretonneux, France) containing fresh MG-buffer (1:80, 1 mg of biopsy to 80 µL of buffer). MG-buffer was comprised of the following: 10 % glycerol, 20 mM sodium-pyrophosphate, 150 mM NaCl, 50 mM 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES, pH 7.5), 1 % NP-40, 20 mM β-glycerophosphate, 2 mM Na_3VO_4, 10 mM Na-F, 2 mM phenylmethanesulfonyl fluoride (PMSF), 1 mM ethylenediaminetetraacetic acid (EDTA, pH 8.0), 1 mM ethylene glycol-bis(2-

aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA, pH 8.0), 10 µg mL^{-1} apoprotinin, 10 µg mL^{-1} leupeptin, 3 mM benzamidine. Samples were homogenized for 2 x 20 s at 6500 rpm with a 5 s pause (Precellys®24, Bertin Technologies, Montigny-le-Bretonneux, France). Thereafter, samples were turned end over end for 1 h at 4 °C, centrifuged at 16500 rpm for 30 min at 4 °C and the supernatant (lysate) was aliquoted out and used for further analysis. Total protein concentration in each sample was determined using a bovine serum albumin (BSA) standard kit (Pierce, Rockford, IL, USA).

Citrate Synthase (CS) activity

CS activity was quantified fluorometrically at 412 nm and 25 °C according to the manufacturer (CS assay kit, Sigma-Aldrich, St. Louis, MO, USA) and as previously described (Jacobs et al. 2012).

Immunoblotting of antioxidant enzymes

Abundance of catalase and mitochondrial superoxide dismutase 2 (SOD2) was determined in the homogenized samples. Homogenates were solubilized in 4 x sample buffer containing 4 x Laemmli sample buffer (161-0747, Bio-Rad, Hercules, CA, USA) and β-mercaptoethanol (161-0710, Bio-Rad, Hercules, CA, USA) and heated up to 95 °C for 5 min. Protein (15 µg per well) was added on 12.5 % precast polyacrylamide gels (12.5 % Criterion™ Tris-HCl Gel, 345-0016, Bio-Rad, Hercules, CA, USA) and separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE, 10 x premixed electrophoresis buffer, containing 25 mM Tris, 192 mM glycine, 0.1 % SDS, pH 8.3, Bio-Rad, Hercules, CA, USA). The separated polypeptides were transferred to a PVDF membrane by the use of Trans-Blot Turbo Transfer System (Bio-Rad, Hercules, CA, USA) and blocked in tris-buffered saline and 0.1 % Tween-20 (TBST) with 5 % non-fat milk. Membranes were cut and incubated overnight with rabbit polyclonal antibodies against catalase (ab16731, Abcam, Cambridge, MA, USA) and SOD2 (ab13534, Abcam, Cambridge, MA, USA) at a dilution of 1:1000. Anti-GAPDH was used as a loading control (ab9484, Abcam, Cambridge, MA, USA). After washing 3 x 10 min with TBST, membranes were incubated with the appropriate anti-rabbit IgG (W4011, Promega, Madison, WI, USA) or anti-mouse IgG (W4021, Promega, Madison, WI, USA) horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. The membranes were again washed and incubated for 5 min each with a chemiluminescence detection marker (Luminata Classico Western HRP Substrate, WBLUC0100, Merck Millipore, Darmstadt, Germany) and detected by Fujifilm Image Analyser LAS-4000 (GE Healthcare, Buckinghamshire, United Kingdom). Relative intensities

of the protein bands were digitally quantified by using ImageJ (NIH, Bethesda, MD, USA) and normalized to GAPDH.

Marker of oxidative stress

Protein carbonyl groups represent one possible biomarker of oxidative stress (Dalle-Donne et al. 2003). Total protein carbonyl content in muscle lysates was determined according to the manufacturer's recommendations using a commercially available kit (STA-308, OxiSelect Protein Carbonyl Immunoblot Kit, Cell Biolabs, San Diego, CA, USA). Values were expressed in arbitrary units (AU) and normalized to mg protein content.

Statistics

All data are presented as mean ± SD in text and figures. For all statistical evaluations, a value of $P < 0.05$ was considered significant. The statistical analysis was conducted using the software SPSS Statistics 22.0 (SPSS, Chicago, IL, USA). Three different statistical models were used for analysis. After verifying normal distribution of the data, unpaired samples *t*-tests were used to test the null hypothesis stating no difference between YNG and OLD for GE_{rel}, GE_{100}, DE, EC, RER_{rel}, RER_{100}, % ST and % FT fibers, CSA of ST and FT fibers, CS activity, Mit_{EFF}, ETF_{EFF}, mass-specific and mitochondrial-specific (normalized to CS activity) respiratory capacities as well as for oxidative stress and antioxidant enzyme activity. Pearson's Correlation Coefficients were calculated to test any relation between GE_{rel} and DE, respectively, and the according variables. If appropriate conditions were satisfied and there was no difference between groups, an analysis of covariance was conducted to measure the associations between GE_{rel} and % ST, % FT, CSA of ST and FT fibers, CS activity, P, Mit_{EFF}, ETF_{EFF} (mass- and mitochondrial-specific), leg_{total} and leg_{lean} among the two age groups. These relations were only conducted for GE_{rel}, as GE reflects the efficiency of the entire human body in action and does not exclude BMR (Ettema and Loras 2009) and we considered the relative calculation to be more significant when comparing age differences.

Results

All participants completed the skeletal muscle biopsy procedure, the DXA measurement and the incremental cycling exercise test. There was no difference in age-adjusted $\dot{V}O_{2peak}$ between groups (Table 1), indicating appropriate matching of OLD and YNG participants according to fitness level.

Exercise efficiency and age

EE was assessed by the use of three different parameters: GE, DE and EC. Beyond, GE was calculated at absolute (GE_{100}) and relative (GE_{rel}) exercise intensities (Table 1). Correlations between EE and other variables were only calculated for GE_{rel} as previously explained. GE_{rel}, GE_{100} and EC were 18, 25 and 29 % higher ($P < 0.01$) in OLD compared to YNG, whereas DE was not different ($P = 0.149$). RER_{rel} and RER_{100} were lower in OLD ($P < 0.01$).

Lean mass and fiber type distribution

There was no difference in leg_{total}, leg_{lean} ($P = 0.457$, $P = 0.118$, respectively) or in muscle fiber type distribution ($P = 0.109$) between the two groups (Table 1). CSA of FT fibers tended to be lower ($P = 0.052$) in OLD whereas CSA of ST fibers did not differ (Table 2). There was no correlation between CSA of ST or FT fibers, respectively, and GE_{rel} when data were pooled ($r = -0.228$; $P = 0.295$; $r = -0.195$; $P = 0.374$). Moreover, there was no overall correlation between GE_{rel} and leg_{total} ($r = -0.153$, $P = 0.487$), leg_{lean} ($r = -0.203$, $P = 0.352$), % ST ($r = 0.104$, $P = 0.636$) and % FT ($r = -0.104$, $P = 0.637$). Neither leg_{total} nor leg_{lean} had an influence on the higher GE_{rel} with age ($P = 0.754$; $P = 0.976$). Similarly fiber type distribution and CSA of ST or FT fibers, respectively, did not have any influence on GE_{rel} ($P = 0.564$; $P = 0.310$; $P = 0.084$).

Skeletal muscle mitochondrial function and CS activity

P_{ETF} (mass-specific respiration) was 56 % higher ($P < 0.001$) and P_{ETF}/CS (mitochondrial-specific) was 38 % higher ($P < 0.01$) in OLD (Fig.1). In contrast, P_{CII} and P_{CII}/CS were 20 and 36 % lower ($P < 0.01$) in OLD (Fig.1). All other respiratory states were similar in OLD and YNG; also Mit_{EFF} and ETF_{EFF} did not differ ($P = 0.571$ and $P = 0.230$, respectively) between groups (Table 2). Furthermore, CS activity was not different between the two groups ($P = 0.098$, Table 2), suggesting similar mitochondrial content. Pooled P_{ETF} ($r = 0.559$, $P < 0.01$)

and P_{ETF}/CS (r = 0.701, P < 0.001, Fig. 2) correlated with GE_{rel}, which could explain higher GE_{rel} in OLD. Contrary, GE_{rel} did not correlate with pooled P or P/CS (r = 0.108, P = 0.625; r = 0.142, P = 0.519). There was also no correlation between GE_{rel} and Mit_{EFF} or Mit_{EFF}/CS (r = 0.211, P = 0.335; r = 0.189, P = 0.388), GE_{rel} and ETF_{EFF} or ETF_{EFF}/CS (r = −0.015, P = 0.944; r = 0.063, P = 0.776) nor did GE and CS activity correlate (r = 0.022, P = 0.921) when the age difference was disregarded. DE did not correlate with any of the reported variables, except from ETF_{EFF} and ETF_{EFF}/CS, (r = 0.421, P < 0.05; r = 0.444, P < 0.05). Additionally, when the two age groups were considered, there was no correlation between GE_{REL} and P or P/CS (P = 0.847; P = 0.073). Similarly, Mit_{EFF} and Mit_{EFF}/CS and ETF_{EFF} and ETF_{EFF}/CS, respectively, had no influence on the age-dependent increase in GE_{REL} (P = 0.446 and P = 0.110; P = 0.429 and P = 0.128). Lastly, also the similar CS activity did not have an influence on the higher GE_{rel} in OLD compared to YNG (P = 0.267).

Oxidative stress and antioxidant enzyme activity

There was no difference (P = 0.852) in catalase content between the two groups (Table 2). However, levels of SOD2 in OLD were higher (103 %, P < 0.01), suggesting an elevated level of antioxidant enzyme activity in OLD (Table 2). To indirectly determine the level of oxidative stress in skeletal muscle tissue, the content of carbonyl groups was determined (Table 2), which revealed higher oxidative stress in OLD (27 %, P < 0.05).

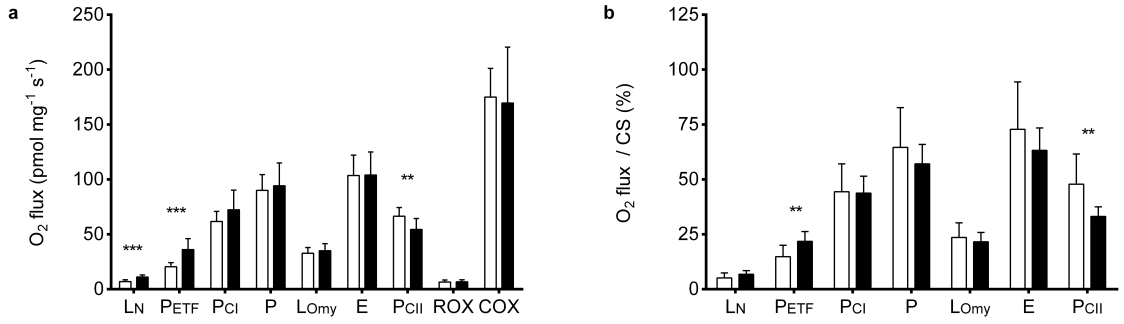

Fig. 1 (a) Mass-specific mitochondrial respiratory capacity and **(b)** mitochondrial-specific respiratory capacity (normalized to citrate synthase (CS) activity) in young (YNG, white bars) and old (OLD, black bars) participants. L_N, leak respiration without adenylates; P_{ETF}, fatty acid oxidative capacity; P_{CI}, respiratory capacity of complex I; P, oxidative phosphorylation capacity; L_{Omy}, oligomycin-induced leak respiration; E, electron transport system capacity; P_{CII}, respiratory capacity of complex II; ROX, residual oxygen consumption; COX, respiratory capacity of complex IV. Values are mean ± SD. **P* < 0.05; ***P* < 0.01; *** *P* < 0.001. n_{YNG} = 11, n_{OLD} = 12

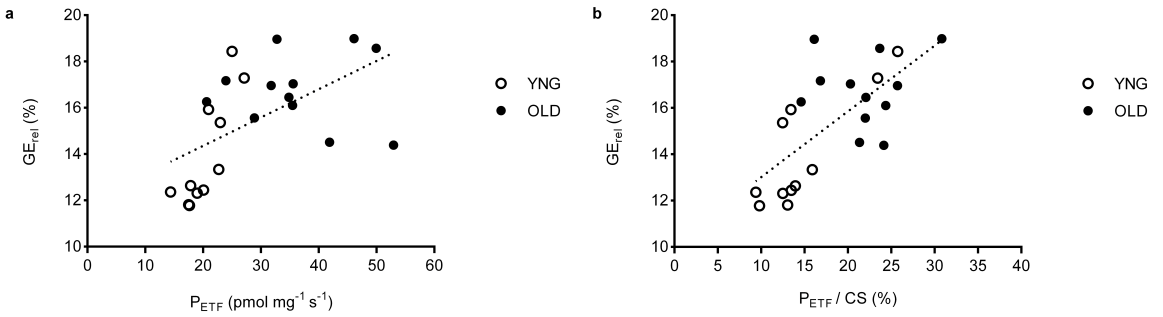

Fig. 2 Correlations irrespective of age (dotted line) between gross efficiency (GE_{rel}) and **(a)** mass-specific maximal electron flow through electron-transferring flavoprotein (P_{ETF}, r = 0.559, P < 0.01) and **(b)** mitochondrial-specific maximal electron flow through electron-transferring flavoprotein (P_{ETF}/CS, r = 0.701, P < 0.001). YNG, young participants; OLD, old participants. n_{YNG} = 11, n_{OLD} = 12

Table 1 Characteristics of young (YNG) and old (OLD) participants

	YNG	OLD	*P*-value
age (yr)	27 ± 4	66 ± 4	< 0.001
weight (kg)	80 ± 13	85 ± 14	0.450
leg_{total} (kg)	28.3 ± 5.1	26.8 ± 4.7	0.457
leg_{lean} (kg)	21.7 ± 3.7	19.4 ± 2.9	0.118
P_{peak} (W)	253 ± 50	184 ± 25	< 0.01
absolute $\dot{V}O_{2peak}$ (L min^{-1})	3.7 ± 0.6	2.5 ± 0.4	< 0.001
relative $\dot{V}O_{2peak}$ (mL min^{-1} kg^{-1})	46.7 ± 5.2	30.3 ± 4.3	< 0.001
age-adj. $\dot{V}O_{2peak}$ (mL min^{-1} kg^{-1})	46.7 ± 5.2	46.3 ± 4.3	0.875
RER_{rel} (-)	0.95 ± 0.07	0.80 ± 0.06	< 0.001
RER_{100} (-)	0.99 ± 0.11	0.86 ± 0.08	< 0.01
GE_{rel} (%)	14.0 ± 2.4	16.7 ± 1.5	< 0.01
GE_{100} (%)	13.5 ± 1.7	17.4 ± 1.6	< 0.001
DE (%)	22.1 ± 4.1	25.4 ± 6.3	0.149
EC (kJ L^{-1})	2.7 ± 0.2	3.6 ± 0.4	< 0.001

Values for YNG and OLD are represented as means ± SD, n_{YNG} = 11, n_{OLD} = 12. Leg_{total}, total mass of the leg; leg_{lean}, lean soft tissue mass of the leg; P_{peak}, peak power; absolute $\dot{V}O_{2peak}$, absolute peak oxygen uptake; relative $\dot{V}O_{2peak}$, peak oxygen uptake per kg body weight; age-adj. $\dot{V}O_{2peak}$, age-adjusted peak oxygen uptake per kg body weight; RER_{rel}, respiratory exchange ratio at relative power stages; RER_{100}, respiratory exchange ratio at 100 W; $GE_{rel,}$ gross efficiency at relative power stages; GE_{100}, gross efficiency at 100 W; DE, delta efficiency; EC, exercise economy

Table 2 Skeletal muscle properties in young (YNG) and old (OLD) participants

	YNG	OLD	*P*-value
CS (nmol mg^{-1} min^{-1})	144.8 ± 25.7	166.4 ± 33.3	0.098
Mit_{EFF} (%)	32.9 ± 8.2	34.5 ± 4.1	0.571
Mit_{EFF}/CS (%)	23.4 ± 7.0	21.6 ± 5.4	0.486
ETF_{EFF} (%)	34.8 ± 5.9	31.8 ± 5.9	0.230
ETF_{EFF}/CS (%)	25.1 ± 7.8	20.3 ± 6.7	0.129
ST fibers (%)	53.4 ± 10.9	61.3 ± 11.7	0.109
FT fibers (%)	46.6 ± 10.9	38.7 ± 11.7	0.109
CSA_{ST} (μm^2)	4945 ± 1402	4407 ± 1058	0.309
CSA_{FT} (μm^2)	5246 ± 1383	4122 ± 1233	0.052
Catalase (AU)	1.15 ± 0.97	1.08 ± 0.95	0.852
SOD2 (AU)	0.64 ± 0.40	2.02 ± 1.33	< 0.01
Protein carbonyl (AU)	0.09 ± 0.03	0.12 ± 0.02	< 0.05

Values for YNG and OLD are represented as means ± SD, n_{YNG} = 11, n_{OLD} = 12. CS, citrate synthase; Mit_{EFF}, mitochondrial coupling efficiency; ETF_{EFF}, coupling efficiency of fat respiration; ST fibers, slow-twitch fibers; FT fibers, fast-twitch fibers; CSA_{ST}, cross-sectional are of slow-twitch fibers; CSA_{FT}, cross-sectional area of fast-twitch fibers; SOD2, mitochondrial superoxide dismutase 2

Discussion

This study demonstrates that EE is higher in OLD compared to YNG at similar levels of fitness. P_{ETF} was higher in OLD and positively correlated with EE. This higher lipid oxidative capacity with age was attended by lower RER at submaximal power in OLD. Moreover, DE was positively correlated with ETF_{EFF}. Contrary to our hypothesis, fiber type distribution did not differ between groups and, hence, could not explain the higher EE in OLD. However, CSA of FT fibers tended to be lower in OLD which could indicate a higher reliance on the more efficient ST fibers with age. The present findings furthermore demonstrate that the higher EE in OLD is not related to CS activity, P, Mit_{EFF}, or ETF_{EFF}, irrespective whether the age groups were pooled or not.

Exercise efficiency and age

In support of previous studies (Venturelli et al. 2013), the present findings demonstrate higher EE in older individuals, when GE and EC were used as measures for EE. Our findings extend previous work by considering the age-dependent decrease of $\dot{V}O_{2peak}$ (Wilson and Tanaka 2000) when eliminating the confounding factor of fitness level. Contrary, Hopker et al. (2013) reported lower GE in trained old vs. trained young participants. However, considering GE at 100 and 150 W of their untrained old and untrained young participants, whose $\dot{V}O_{2peak}$ (young ≈ 47 mL min^{-1} kg^{-1}; old ≈ 34 mL min^{-1} kg^{-1}) are in line with the present (YNG ≈ 47 mL min^{-1} kg^{-1}; OLD ≈ 30 mL min^{-1} kg^{-1}), a higher GE in the old untrained individuals becomes visible but was not statistically tested for (Table 2, Hopker et al. 2013). Since the relationship between caloric output and power has been reported to be linear or even slightly exponential, constant or decreasing EE with increasing power, respectively, have been implied (Gaesser and Brooks 1975). In general, the reported (Hopker et al. 2013) values for GE were slightly higher (100 W: young ≈ 16 % and old ≈ 17 %; 150 W: young ≈ 18 % and old ≈ 19 %) than the values in the present study (YNG ≈ 14 % and OLD ≈ 17 %). This might be explained by the different calculation approach for GE, as we considered unmodified absolute and relative values (100 W and mean of 50, 60 and 70 % $\dot{V}O_{2peak}$, respectively) instead of absolute rescaled data (Hopker et al. 2013).

Other studies have reported a decrease in EE with age during walking and running (Hortobagyi et al. 2011; Ortega and Farley 2007). However, as stated by the authors (Hortobagyi et al. 2011; Ortega and Farley 2007), the reported decrease in walking efficiency in old participants could likely be the consequence of the utilization of ancillary muscles and

may hence not be a result of muscular metabolic efficiency *per se*. Therefore the direct comparison between studies utilizing walking efficiency with those investigating cycling efficiency should be taken with some caution. Moreover, numerous studies did not control for activity or fitness level, which may further complicate a direct comparison (Russ and Kent-Braun 2004). There are, however, findings demonstrating lower EE in well-trained old men during cycling (Sacchetti et al. 2010), which are contradictory to the present study. Discrepancies between studies could be due to biological differences such as anthropometric measures (Bramble and Lieberman 2004) but might also be explained by the training level of the participants, as they included highly trained cyclists (Sacchetti et al. 2010). The comparison of EE of Masters-level to younger elite cyclists could induce a bias since normative data from elite young cyclists should not be directly applied to evaluate the fitness level of the older (Peiffer et al. 2008). Additionally, cyclists have been reported to be able to increase their EE throughout their career (Coyle 2005). Nevertheless, it has been demonstrated that age-related changes in EE in untrained individuals can be reversed or enhanced with exercise training (Amati et al. 2008; Conley et al. 2013; Woo et al. 2006). Therefore, in support of our findings, cycling-specific EE can be preserved (Peiffer et al. 2008) or even enhanced with age (Kent-Braun and Ng 2000; Lanza et al. 2005; Venturelli and Richardson 2013; Venturelli et al. 2013).

To further solidify differences in EE with advances in age, we evaluated EE not only by considering absolute and relative GE but also DE and EC. DE, however, did not differ between YNG and OLD. A possible explanation for higher GE and EC but unchanged DE in OLD could be an age-dependent alteration in BMR. In contrast to GE, which reflects the efficiency of the entire human organism, and therefore also includes the influence of basal metabolism (Ettema and Loras 2009), DE is calculated by the change in energy expended relative to the change in actual work accomplished, whereby the influence of BMR is subtracted (Coyle et al. 1992; Gaesser and Brooks 1975). The validity of BMR subtraction, however, has been questioned (Ettema and Loras 2009; Moseley and Jeukendrup 2001) and GE and EC are both suggested more reliable measures of whole body efficiency than DE, which also displays a higher day-to-day variability (Ettema and Loras 2009; Moseley and Jeukendrup 2001). Accordingly, smaller changes in EE can be detected considering GE but not DE (Moseley and Jeukendrup 2001). Beyond, it seems unlikely that there was a difference in BMR between the two age groups, since on the one hand DE tended to be higher in OLD (25.4 ± 6.3 vs. 22.1 ± 4.1 %, P = 0.149) and on the other hand neither weight and lean body mass, which are among the main predictors of BMR (Cunningham 1980) nor leg_{total} and leg_{lean} differed between OLD and YNG.

Exercise efficiency and skeletal muscle fiber type distribution

As EE has previously been shown to be related to the percentage of ST fibers in some studies (Coyle et al. 1992; Mogensen et al. 2006), we initially aimed to investigate whether this relationship was affected by training state and aging. However, skeletal muscle fiber type distribution was not related to GE and the higher GE in OLD did not correlate with fiber type distribution. These findings are in accordance with some studies (Amati et al. 2008; Hopker et al. 2013) but in contrast to others (Coyle et al. 1992; Mogensen et al. 2006). The discrepancy between the present findings and those who have reported a correlation between EE and skeletal muscle phenotype (Coyle et al. 1992; Mogensen et al. 2006) might be due to the fact that they included endurance trained cyclists and the percentage of ST fibers is related to the number of years of endurance training (Coyle et al. 1992). Thus, contrary to our hypothesis, fiber type distribution could not explain the observed difference in GE with age, irrespective if pooled or unpooled data were used. Moreover, fiber type distribution did not differ between the age groups, which is in agreement with previous findings (Hopker et al. 2013; Proctor et al. 1995; Purves-Smith et al. 2014). A possible explanation for the unchanged relative fiber type distribution with age could be the level of fitness of the included subjects. In support herefore leg_{total} and leg_{lean} did not differ between OLD and YNG, indicating an absence of age-dependent skeletal muscle atrophy if fitness level is taken into account. Others have also reported an unaltered skeletal muscle phenotype in sedentary (Coggan et al. 1992), recreationally active and trained (Proctor et al. 1995) old. However, in the old, cross-sectional area occupied by ST fibers was higher than that occupied by FT fibers (Coggan et al. 1992; Proctor et al. 1995). The atrophy of FT fibers with age might again be explained by inactivity rather than aging *per se* (Coggan et al. 1992; Proctor et al. 1995). Alternatively, the decline in $\dot{V}O_{2peak}$ with age (Wilson and Tanaka 2000) could limit absolute training intensity and thereby lead to atrophy of FT fibers, which in turn seems to be compensated for by increased ST area (Coggan et al. 1992). Hence, the physical activity level of the study participants could likely have contributed to the inconsistent findings regarding the direction and magnitude of changes in fiber type with age (Purves-Smith et al. 2014). Other possible explanations for the discrepant outcomes that may generate the hypothesis of an age-dependent shift in fiber type are reviewed in detail by Purves-Smith et al. (2014) and include: 1) the age of the participants. Most studies focus on the ages of 65 to 75 yr, which are ages not typically associated with mobility impairment and muscle atrophy; 2) different behaviour of different muscles with aging and a potential dependence of the shift in fiber type on its initial distribution; 3) the age-dependent increase in myosin heavy-chain (MHC) coexpressing fibers due to denervation (Rowan et al. 2012).

The present study reported no difference in CSA of ST fibers and only a tendency towards lower are of FT fibers with higher age. Since the OLD and YNG were compared at the same relative exercise intensity and muscle mass was similar, the OLD exercised at lower absolute power outputs, which may have facilitated a higher reliance on ST fibers in OLD compared to YNG, reflected by the tendency to lower area of FT fibers. Therefore, despite similar fiber type distribution a potentially higher recruitment of ST fibers could partly explain higher EE with advancing age.

Exercise efficiency and mitochondrial function

The activity of CS in the present study did not differ between the two groups. Thus, maintenance of CS activity with age indicates that mitochondrial enzyme activity and mitochondria populations are not affected by the aging process *per se*. This is in contrast to some studies that show a decline in CS activity with increasing age (Coggan et al. 1992; Conley et al. 2000). However, when fitness level was considered or controlled for, CS activity did not differ between old and young (Gram et al. 2014; Lanza et al. 2005; Larsen et al. 2012a; Rasmussen et al. 2003), which is in line with our findings. In support, Broskey et al. (2013) demonstrated that aging does not affect mitochondrial biogenesis and that observed decreases in mitochondrial function with age are likely due to decreased physical activity. The unchanged CS activity suggests at least maintained skeletal muscle oxidative capacity and, if anything, CS activity tended to be even higher (P = 0.098) in OLD, which accords with the findings of Larsen et al. (2012a). The present findings, however, could not determine a positive association between GE and CS activity.

In addition, we observed similar P and Mit_{EFF} in the two age groups. In support, also other investigators observed similar absolute maximum capacity of mitochondrial ATP synthesis between old and young study participants (Gram et al. 2014; Kent-Braun and Ng 2000). Similarly, maximal mitochondrial respiratory capacity in young (≈ 23 yr) and middle-aged (≈ 53 yr) men at similar $\dot{V}O_{2peak}$ did not differ, however, in contrast to our findings, mitochondrial respiratory capacity per mitochondrion was lower in the middle-aged (Larsen et al. 2012a). Yet, this discrepancy may be explained by the disregard of the age-related decline in $\dot{V}O_{2peak}$ in the former study (Larsen et al. 2012a), as also others observed no difference in intrinsic mitochondrial capacity with age (Hutter et al. 2007; Lanza et al. 2005; Rasmussen et al. 2003). In contrast, there are studies reporting reductions in mitochondrial respiratory capacity and oxidative capacity with age (Conley et al. 2000). However, none of these controlled for level of fitness, indicating that the results may rather be related to age-dependent lifestyle changes than to the aging process itself (Conley et al. 2013; Gram et al. 2014; Russ and

Kent-Braun 2004). Additionally, as discussed in detail by Rasmussen et al. (2003), the observed decline in mitochondrial function with age may also be likely due to heavy preparational damage. Collectively, by preserving muscle mass and function with age, mitochondrial biogenesis, morphology and function may remain stable. Accordingly, these data further suggest preserved mitochondrial respiratory function with age.

Moreover, the present respirometry data suggest a higher capacity but not a higher efficiency of mitochondrial fat oxidation with age (higher P_{ETF} and P_{ETF}/CS in OLD vs. YNG), which may be reflected in the lower RER values of the OLD at submaximal work rates. These findings are confirmatory to the work of Amati et al. (2008) and Rimbert et al. (2004), who reported increased reliance on fat oxidation in old individuals during moderate-intensity exercise after exercise training (Amati et al. 2008) and unaltered fatty acid oxidative capacity with age (Rimbert et al. 2004), respectively. Noteworthy, we observed a positive relationship between GE_{rel}, P_{ETF} and P_{ETF} /CS activity and DE, ETF_{EFF} and ETF_{EFF}/CS, respectively. Hence, at least part of the increase in EE between OLD and YNG could be related to a higher capacity and/or efficiency for/of fatty acid oxidation and a maintained capacity for maximal mitochondrial respiration. Analysing the graph of this relation (Fig. 2), it should however be noted that this relation could be due to a stronger correlation between GE_{rel} and P_{ETF} of the YNG compared to the OLD. Nonetheless, Lanza et al. (2007) provided evidence of a higher metabolic economy in old vs. young human skeletal muscle in face of comparable mitochondrial respiratory capacity. This finding is in agreement with an age-dependent preferential reliance on oxidative phosphorylation, determined with magnetic resonance spectroscopy measurements (Lanza et al. 2007). The similar maximal capacity for mitochondrial ATP synthesis but higher preference for ATP production from oxidative phosphorylation (Lanza et al. 2007) together with the higher capacity for fat oxidation observed in the present study could be attributed to a higher ST recruitment (as previously discussed) and hence could account for an increase in EE. Contrary, there are findings indicating reduced fatty acid oxidation with age (Sial et al. 1996). However, fat oxidation rates at rest were similar in old and young individuals and higher in old at similar relative intensities (Sial et al. 1996), which reflects the design of the present study. Collectively, further investigations concerning metabolism, efficiency and the process of age are required.

Oxidative stress and aging

Augmented oxidative stress has been suggested to play an important role in the aging process, potentially leading to deficiencies in mitochondrial function. However, as discussed, we did not observe a decline in mitochondrial function. Furthermore, our data revealed higher

levels of SOD2 and carbonyl groups. The higher levels of antioxidant enzyme activity in OLD, represented by SOD2, may have been the result of a compensation for the larger number of carbonyl groups that represent a reliable marker for oxidative stress (Dalle-Donne et al. 2003). Aged human muscle has been reported to show preserved mitochondrial function despite higher reactive oxygen species (ROS) production, estimated by dihydroethidium-staining (Hutter et al. 2007). Collectively, the mechanisms for the higher levels of markers for oxidative stress despite maintained mitochondrial function remain to be further investigated.

Limitations and outlook

The present study bears the limits of a cross-sectional analysis, but was preferred over a longitudinal study due to time management. Another limitation could be the use of protocols with relatively short stage durations (120 s), which could have led to an overestimation of EE in the OLD as older adults demonstrate slower $\dot{V}O_2$ kinetics during non-steady-state conditions, implying greater EE than younger counterparts (Gravelle et al. 2012). Under steady-state conditions however, there were no changes in $\dot{V}O_2$ kinetics and hence no differences in EE between groups (Gravelle et al. 2012). Anyhow, slowing of $\dot{V}O_2$ kinetics in older individuals is to a large extent related to decreased physical activity and aerobic fitness with age (Berger et al. 2006; Grey et al. 2015). Moreover, moderately active older individuals reported similar $\dot{V}O_2$ kinetics to young counterparts (Chilibeck et al. 1998). Matching participants by fitness level and using the last 30 s for the calculation of energy expenditure should have enabled a valid determination of EE, which was randomly controlled. As pedal cadence was freely chosen between 60 and 90 rpm, this could also have limited the present results (Sacchetti et al. 2010). However, for this spectrum of cadences and considering the chance of choosing the convenient cadence, a major influence of pedal rate on EE in this study seems unlikely. For further improvements, also other factors could be considered in future studies: 1) Musculoskeletally less flexible distance runners tend to be more economical than more flexible runners, possibly because of the energy-efficient function of the elastic components in the muscles and tendons during the stretch-shortening cycle (Trehearn and Buresh 2009). Aging on the other hand has been associated with a decline in limb flexibility (Fukuchi et al. 2014), which may be an explanation for the increase in EE and which would be rather simple to assess; 2) Another aspect that could be investigated in future studies could be the potential age-related change in capillary morphology and its influence on EE, as capillary morphology may be related to whole-body $\dot{V}O_2$ kinetics.

Conclusions

The present study extends previous studies in the field by accounting for the age-related decrease in $\dot{V}O_{2peak}$. Furthermore, EE was higher in OLD vs. YNG after controlling for % ST, % FT, CSA of ST and FT fibers, CS activity, P, Mit_{EFF} or ETF_{EFF}. These finding indicate that impairments originally ascribed to age could be related to the negative effects of physical inactivity. Additionally, EE was positively correlated to P_{ETF}, suggesting that older individuals may have the possibility to rely more on fatty acid oxidation and are therefore more efficient. The higher capacity of fatty acid oxidation was further reflected by a lower RER with age. A potentially higher recruitment of ST fibers mirrored by a tendency towards lower FT fiber cross-sectional area could partly explain the higher reliance on fatty acid oxidation and thereby the higher EE with increasing age. The energetic needs of the older study participants were possibly met with a smaller perturbation of intracellular metabolism and may have therefore led to higher EE. Additional influencing factors on EE and the interdependency of age remain to be investigated, as EE is a key determinant of exercise capacity and thus mobility of the elderly. Increases in EE may have considerable implications for older individuals, who are at greater risk for functional impairments. Enhanced efficiency during physical activity would imply less energy requirements in the activities of daily living, which could be of clinical relevance.

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Conflict of interest

The authors declare that they have no conflict of interest.

References

Amati F, Dube JJ, Shay C, Goodpaster BH (2008) Separate and combined effects of exercise training and weight loss on exercise efficiency and substrate oxidation. J Appl Physiol (1985) 105:825-831. doi:10.1152/japplphysiol.90384.2008

Berger NJ, Rittweger J, Kwiet A, Michaelis I, Williams AG, Tolfrey K, Jones AM (2006) Pulmonary O2 uptake on-kinetics in endurance- and sprint-trained master athletes. Int J Sports Med 27:1005-1012. doi:10.1055/s-2006-923860

Bramble DM, Lieberman DE (2004) Endurance running and the evolution of Homo. Nature 432:345-352. doi:10.1038/nature03052

Broskey NT, Daraspe J, Humbel BM, Amati F (2013) Skeletal muscle mitochondrial and lipid droplet content assessed with standardized grid sizes for stereology. J Appl Physiol (1985) 115:765-770. doi:10.1152/japplphysiol.00063.2013

Chilibeck PD, Paterson DH, McCreary CR, Marsh GD, Cunningham DA, Thompson RT (1998) The effects of age on kinetics of oxygen uptake and phosphocreatine in humans during exercise. Exp Physiol 83:107-117.

Coggan AR, Spina RJ, King DS, Rogers MA, Brown M, Nemeth PM, Holloszy JO (1992) Histochemical and enzymatic comparison of the gastrocnemius muscle of young and elderly men and women. J Gerontol 47:B71-76.

Conley KE, Jubrias SA, Cress ME, Esselman PC (2013) Elevated energy coupling and aerobic capacity improves exercise performance in endurance-trained elderly subjects. Exp Physiol 98:899-907. doi:10.1113/expphysiol.2012.069633

Conley KE, Jubrias SA, Esselman PC (2000) Oxidative capacity and ageing in human muscle. J Physiol 526 Pt 1:203-210.

Coyle EF (2005) Improved muscular efficiency displayed as Tour de France champion matures. J Appl Physiol (1985) 98:2191-2196. doi:10.1152/japplphysiol.00216.2005

Coyle EF, Sidossis LS, Horowitz JF, Beltz JD (1992) Cycling efficiency is related to the percentage of type I muscle fibers. Med Sci Sports Exerc 24:782-788.

Cunningham JJ (1980) A reanalysis of the factors influencing basal metabolic rate in normal adults. Am J Clin Nutr 33:2372-2374.

Dalle-Donne I, Rossi R, Giustarini D, Milzani A, Colombo R (2003) Protein carbonyl groups as biomarkers of oxidative stress. Clin Chim Acta 329:23-38.

Ettema G, Loras HW (2009) Efficiency in cycling: a review. Eur J Appl Physiol 106:1-14. doi:10.1007/s00421-009-1008-7

Fukuchi RK, Stefanyshyn DJ, Stirling L, Duarte M, Ferber R (2014) Flexibility, muscle strength and running biomechanical adaptations in older runners. Clin Biomech (Bristol, Avon) 29:304-310. doi:10.1016/j.clinbiomech.2013.12.007

Gaesser GA, Brooks GA (1975) Muscular efficiency during steady-rate exercise: effects of speed and work rate. J Appl Physiol (1985) 38:1132-1139.

Gnaiger E (2009) Capacity of oxidative phosphorylation in human skeletal muscle: new perspectives of mitochondrial physiology. Int J Biochem Cell Biol 41:1837-1845. doi:10.1016/j.biocel.2009.03.013

Gram M, Vigelso A, Yokota T, Hansen CN, Helge JW, Hey-Mogensen M, Dela F (2014) Two weeks of one-leg immobilization decreases skeletal muscle respiratory capacity equally in young and elderly men. Exp Gerontol 58:269-278. doi:10.1016/j.exger.2014.08.013

Gravelle BM, Murias JM, Spencer MD, Paterson DH, Kowalchuk JM (2012) Adjustments of pulmonary O2 uptake and muscle deoxygenation during ramp incremental exercise and constant-load moderate-intensity exercise in young and older adults. J Appl Physiol (1985) 113:1466-1475. doi:10.1152/japplphysiol.00884.2011

Grey TM, Spencer MD, Belfry GR, Kowalchuk JM, Paterson DH, Murias JM (2015) Effects of age and long-term endurance training on VO2 kinetics. Med Sci Sports Exerc 47:289-298. doi:10.1249/MSS.0000000000000398

Hopker JG, Coleman DA, Gregson HC, Jobson SA, Von der Haar T, Wiles J, Passfield L (2013) The influence of training status, age, and muscle fiber type on cycling efficiency and endurance performance. J Appl Physiol (1985) 115:723-729. doi:10.1152/japplphysiol.00361.2013

Hortobagyi T, Finch A, Solnik S, Rider P, DeVita P (2011) Association between muscle activation and metabolic cost of walking in young and old adults. J Gerontol A Biol Sci Med Sci 66:541-547. doi:10.1093/gerona/glr008

Hutter E, Skovbro M, Lener B, Prats C, Rabol R, Dela F, Jansen-Durr P (2007) Oxidative stress and mitochondrial impairment can be separated from lipofuscin accumulation in aged human skeletal muscle. Aging Cell 6:245-256. doi:10.1111/j.1474-9726.2007.00282.x

Item F, Denkinger J, Fontana P, Weber M, Boutellier U, Toigo M (2011) Combined effects of whole-body vibration, resistance exercise, and vascular occlusion on skeletal muscle and performance. Int J Sports Med 32:781-787. doi:10.1055/s-0031-1277215

Jackman MR, Willis WT (1996) Characteristics of mitochondria isolated from type I and type IIb skeletal muscle. Am J Physiol 270:C673-678.

Jacobs RA, Flück D, Bonne TC, Burgi S, Christensen PM, Toigo M, Lundby C (2013) Improvements in exercise performance with high-intensity interval training coincide with an increase in skeletal muscle mitochondrial content and function. J Appl Physiol (1985) 115:785-793. doi:10.1152/japplphysiol.00445.2013

Jacobs RA, Siebenmann C, Hug M, Toigo M, Meinild AK, Lundby C (2012) Twenty-eight days at 3454-m altitude diminishes respiratory capacity but enhances efficiency in human skeletal muscle mitochondria. FASEB J 26:5192-5200. doi:10.1096/fj.12-218206

Kent-Braun JA, Ng AV (2000) Skeletal muscle oxidative capacity in young and older women and men. J Appl Physiol (1985) 89:1072-1078.

Kyrolainen H, Pullinen T, Candau R, Avela J, Huttunen P, Komi PV (2000) Effects of marathon running on running economy and kinematics. Eur J Appl Physiol 82:297-304. doi:10.1007/s004210000219

Lanza IR, Befroy DE, Kent-Braun JA (2005) Age-related changes in ATP-producing pathways in human skeletal muscle in vivo. J Appl Physiol (1985) 99:1736-1744. doi:10.1152/japplphysiol.00566.2005

Lanza IR, Larsen RG, Kent-Braun JA (2007) Effects of old age on human skeletal muscle energetics during fatiguing contractions with and without blood flow. J Physiol 583:1093-1105. doi:10.1113/jphysiol.2007.138362

Larsen S, Hey-Mogensen M, Rabol R, Stride N, Helge JW, Dela F (2012a) The influence of age and aerobic fitness: effects on mitochondrial respiration in skeletal muscle. Acta Physiol (Oxf) 205:423-432. doi:10.1111/j.1748-1716.2012.02408.x

Larsen S et al. (2012b) Biomarkers of mitochondrial content in skeletal muscle of healthy young human subjects. J Physiol 590:3349-3360. doi:10.1113/jphysiol.2012.230185

Mogensen M, Bagger M, Pedersen PK, Fernstrom M, Sahlin K (2006) Cycling efficiency in humans is related to low UCP3 content and to type I fibres but not to mitochondrial efficiency. J Physiol 571:669-681. doi:10.1113/jphysiol.2005.101691

Montero D, Lundby C (2015) The Effect of Exercise Training on the Energetic Cost of Cycling. Sports Med doi:10.1007/s40279-015-0380-1

Moseley L, Jeukendrup AE (2001) The reliability of cycling efficiency. Med Sci Sports Exerc 33:621-627.

Ortega JD, Farley CT (2007) Individual limb work does not explain the greater metabolic cost of walking in elderly adults. J Appl Physiol (1985) 102:2266-2273. doi:10.1152/japplphysiol.00583.2006

Peiffer JJ, Abbiss CR, Chapman D, Laursen PB, Parker DL (2008) Physiological characteristics of masters-level cyclists. J Strength Cond Res 22:1434-1440. doi:10.1519/JSC.0b013e318181a0d2

Picard M et al. (2010) Mitochondrial functional impairment with aging is exaggerated in isolated mitochondria compared to permeabilized myofibers. Aging Cell 9:1032-1046. doi:10.1111/j.1474-9726.2010.00628.x

Proctor DN, Sinning WE, Walro JM, Sieck GC, Lemon PW (1995) Oxidative capacity of human muscle fiber types: effects of age and training status. J Appl Physiol (1985) 78:2033-2038.

Purves-Smith FM, Sgarioto N, Hepple RT (2014) Fiber typing in aging muscle. Exerc Sport Sci Rev 42:45-52. doi:10.1249/JES.0000000000000012

Rasmussen UF, Krustrup P, Kjaer M, Rasmussen HN (2003) Experimental evidence against the mitochondrial theory of aging. A study of isolated human skeletal muscle mitochondria. Exp Gerontol 38:877-886.

Rimbert V, Boirie Y, Bedu M, Hocquette JF, Ritz P, Morio B (2004) Muscle fat oxidative capacity is not impaired by age but by physical inactivity: association with insulin sensitivity. FASEB J 18:737-739. doi:10.1096/fj.03-1104fje

Rowan SL, Rygiel K, Purves-Smith FM, Solbak NM, Turnbull DM, Hepple RT (2012) Denervation causes fiber atrophy and myosin heavy chain co-expression in senescent skeletal muscle. PLoS One 7:e29082. doi:10.1371/journal.pone.0029082

Russ DW, Kent-Braun JA (2004) Is skeletal muscle oxidative capacity decreased in old age? Sports Med 34:221-229.

Sacchetti M, Lenti M, Di Palumbo AS, De Vito G (2010) Different effect of cadence on cycling efficiency between young and older cyclists. Med Sci Sports Exerc 42:2128-2133. doi:10.1249/MSS.0b013e3181e05526

Sial S, Coggan AR, Carroll R, Goodwin J, Klein S (1996) Fat and carbohydrate metabolism during exercise in elderly and young subjects. Am J Physiol 271:E983-989.

Tevald MA, Foulis SA, Lanza IR, Kent-Braun JA (2010) Lower energy cost of skeletal muscle contractions in older humans. Am J Physiol Regul Integr Comp Physiol 298:R729-739. doi:10.1152/ajpregu.00713.2009

Trehearn TL, Buresh RJ (2009) Sit-and-reach flexibility and running economy of men and women collegiate distance runners. J Strength Cond Res 23:158-162. doi:10.1519/JSC.0b013e31818eaf49

Venturelli M, Richardson RS (2013) Point: skeletal muscle mechanical efficiency does increase with age. J Appl Physiol (1985) 114:1108-1109. doi:10.1152/japplphysiol.01438.2012

Venturelli M, Schena F, Scarsini R, Muti E, Richardson RS (2013) Limitations to exercise in female centenarians: evidence that muscular efficiency tempers the impact of failing lungs. Age (Dordr) 35:861-870. doi:10.1007/s11357-011-9379-1

Wilson TM, Tanaka H (2000) Meta-analysis of the age-associated decline in maximal aerobic capacity in men: relation to training status. Am J Physiol Heart Circ Physiol 278:H829-834.

Woo JS, Derleth C, Stratton JR, Levy WC (2006) The influence of age, gender, and training on exercise efficiency. J Am Coll Cardiol 47:1049-1057. doi:10.1016/j.jacc.2005.09.066

Patients with mitochondrial myopathy exhibit diminished mitochondrial quantity and quality and a shift in skeletal muscle phenotype

in submission

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Running title

Impaired mitochondrial function in patients with mitochondrial myopathy

Abstract

Despite constant progress in the comprehension of biochemical and genetical characteristics in mitochondrial myopathy, the major pathomechanisms and therewith effective treatment approaches remain elusive. Therfore, the aim of the present study was to investigate disease-related impairments in skeletal muscle properties in mitochondrial myopathy. Accordingly, Skeletal muscle biopsies were obtained from six patients with moleculargenetically diagnosed mitochondrial myopathy (one male and five females, 53 ± 9 y) and eight age- and gender-matched healthy controls (two males and six females, 58 ± 14 y) to determine mitochondrial respiratory cacapcity of each single complex, mitochondrial volume density and fiber type distribution. Mitochondrial volume density (4.0 ± 0.5 vs. 5.1 ± 0.8 %) as well as respiratory capacity of each individual complex were lower with mitochondrial myopathy ($P < 0.05$) and accompanied by a higher ($P < 0.001$) proportion of type II fibers (65.2 ± 3.6 vs. 44.3 ± 5.9 %). Additionally, mitochondrial volume density and maximal oxidative phosphorylation capacity positively correlated ($P < 0.05$) to peak oxygen uptake. In conclusion, mitochondrial myopathy leads to impaired mitochondrial quantity and quality and a shift towards more glycolytic skeletal muscle phenotype.

Key words

Bioenergetics; mitochondria; mitochondrial cytopathy; neuromuscular disease

Abbreviations

a-vO_2 diff, arteriovenous oxygen difference; BMC, bone mineral content; CIS, checklist individual strength; CK, phosphocreatine kinase; COPD, chronic obstructive pulmonary disease; COX, respiratory capacity of complex IV; CPEO, chronic progressive external ophthalmoplegia; CSA, cross-sectional area; DXA, dual-energy X-ray absorptiometry; E, electron transport system capacity; ETF_{EFF}, coupling efficiency of fat respiration; ETS, electron transport system; FCCP, carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone; IMF_{VD}, intermyofibrilar volume density; [L^-], blood lactate concentration; LD_{VD}, lipid droplet volume density; L_N, leak respiration without adenylates; L_{Omy}, oligomycin-induced leak respiration; mATPase, myofibrillar adenosinetriphosphatase; MHC, myosin heavy-chain; $Mito_{VD}$, mitochondrial volume density; MM, mitochondrial myopathy; mtDNA, mitochondrial deoxyribonucleic acid; MVC, maximal voluntary contraction torque; nDNA, nuclear deoxyribonucleic acid; P, oxidative phosphorylation capacity; P_{CI}, respiratory capacity of complex I; P_{CII}, respiratory capacity of complex II; P_{ETF}, fatty acid oxidative capacity; ^{31}P-MRS, phosphorus magnetic resonance spectroscopy; P_{peak}, peak power; proBNP, precursorprotein brain natriuretic peptide; $\dot{Q}_{peak}$, peak cardiac outpout; RER, respiratory exchange ratio; ROX, residual oxygen consumption; rpm, revolutions per minute; RRF, ragged-red fibers; RT, room temperature; SATET, subanaerobic threshold exercise test; SDH, succinate dehydrogenase; SS_{VD}, subsarcolemmal mitochondrial density; TEM, transmission electron microscopy; t_{lim}, time to exhaustion; TSH, thyroid stimulating hormone; VAS, visual analogue scale; $\dot{V}O_{2peak}$, peak oxygen uptake; ww, wet weight.

Introduction

Mitochondrial cytopathies represent a heterogeneous group of progressive multisystem disorders with particular affection of the muscle and nervous system (Cohen and Gold, 2001; Schmiedel *et al.*, 2003). Mitochondrial cytopathies are caused by mutations in either mitochondrial DNA (mtDNA) or nuclear DNA (nDNA) encoding for mitochondrial proteins (Wallace, 1999; Tuppen *et al.*, 2010). The irregular distribution of mitochondria in tissues, coexistence of mutated and wild-type mtDNA, so called heteroplasmy, and the widespread range of mutation lead to dysfunction in either one or several mitochondrial metabolic pathways including the respiratory chain and oxidative phosphorylation. These dysfunctions result in a clinical and genetic heterogenity, complexity and severity of symptoms that is characteristic for this syndrome (Di Mauro *et al.*, 1985; Di Mauro, 2004; Schapira, 2006; Moggio *et al.*, 2014). The functional consequence of defective oxidative phosphorylation particularly manifests itself in impaired energy production that amongst others affects skeletal muscle, a tissue with high-energy demand (Rossignol *et al.*, 2000). The affection of skeletal muscle is defined as mitochondrial myopathy and represents a very common manifestation of mitochondrial cytopathies (Di Mauro, 2004), either isolated or combined with other symptoms of mitochondrial cytopathies. Although there has been constant progress in understanding the biochemistry and genetics of mitochondrial myopathy, many of the pathophysiological mechanisms remain unclear (Di Mauro, 2010). Not least due to the incomplete comprehension of these pathomechanisms, proven cures but also simple and effective diagnostic tools are lacking (Larsson and Oldfors, 2001; Di Mauro, 2004, 2010; Hassani *et al.*, 2010; Tuppen *et al.*, 2010).

Patients with mitochondrial myopathy have been characterized by lower peak O_2 uptake ($\dot{V}O_{2peak}$) and work capacity (P_{peak}) in cycling exercise (Vissing *et al.*, 1996; Taivassalo *et al.*, 2002; Taivassalo *et al.*, 2003). The limited whole body oxidative capacity in mitochondrial myopathy patients is likely caused by an impaired ability of the muscle to extract the available O_2 from the blood, as it has been documented invasively by directly determining arteriovenous O_2 difference (a-vO_2 diff) across exercising muscles (Linderholm *et al.*, 1969; Taivassalo *et al.*, 2002) or indirectly (Vissing *et al.*, 1996; Taivassalo *et al.*, 2003) after the assessment of peak cardiac output ($\dot{Q}_{peak}$). The limited O_2 extraction in turn may be explained by impaired oxidative capacity of the mitochondria itself and/or a reduced number of these organelles (Hoppeler *et al.*, 1987) due to the disease-causing mutations or exercise intolerance. Exercise intolerance can be a prominent clinical manifestation of mitochondrial myopathy, leading to relatively low levels of exertion, fast fatigability and a sedentary lifestyle

(Taivassalo and Haller, 2005; Taivassalo *et al.*, 2006). This sedentary lifestyle may further restrict the capacitiy of skeletal muscle for oxidative phosphorylation by the decrease in levels of functional mitochondria (Iqbal *et al.*, 2013). Hence, it remains difficult to distinguish to what extent exercise intolerance is related to impaired mitochondrial oxidative capacity or associated with physical deconditioning due to a sedentary lifestyle. Assessments of muscle oxidative capacity and potential alterations with mitochondrial myopathy have previously been only conducted in case reports or small series (Taivassalo *et al.*, 2003). Thereby, defects of the mitochondrial respiratory chain have been identified polarographically or by enzymatic assays (Morgan-Hughes *et al.*, 1990; Rustin *et al.*, 1994), usually involving complex I or III in adults and complex IV in children (Holt *et al.*, 1989; Enns *et al.*, 2005). Apart from the small series and lacking scientific controls, the poloarographic measurements, however, have been mainly conducted in complex combinations and not independently for each complex (Tarnopolsky and Raha, 2005). Moreover, these more global assessments of mitochondrial function were conducted in muscle homogenates or isolated fibers (Rustin *et al.*, 1994). Mitochondrial isolation and homogenisation procedures however may disrupt the functional structural network and can thereby alter innate mitochondrial characteristics (Picard *et al.*, 2010). Therefore, in vitro measurements of O_2 consumption in permeabilized myofibers preserving innate mitochondrial function present a more promising approach to determine mitochondrial characteristics. Beyond, determination of the respiratory capacity of each individual complex could lead to a better understanding of mitochondrial function and potential mitochondrial-myopathy-related pathomechanisms. Additionally, detailed elucidation of potential pathomechanisms is important not only for the development of rational treatment options but also the initial diagnosis of mitochondrial myopathy. The absence of a consistent clinical phenotype has limited the viability of clinical identification and classification criteria of mitochondrial myopathy so far (Di Mauro, 2004; Tarnopolsky and Raha, 2005; Moggio *et al.*, 2014). Thus, the primary aim of the present study was to determine mitochondrial volume densitiy and respiratory capacity of each individual complex in permeabilized muscle fibers of mitochondrial myopathy patients by implementing high-resolution respirometry. Due to the ascertained moleculargenetic diagnosis of mitochondrial myopathy and the concomitant exercise intolerance, we hypothesized that mitochondrial volume density and respiratory capacity of each single complex in those patients would be decreased compared to age- and gender-matched healthy controls. Furthermore, this study aimed to test whether high-resolution respirometry measurements would represent a promising diagnostic tool leading to enhanced diagnostic and classification criteria of mitochondrial myopathy.

In addition, it has barely been investigated how muscle metabolism and morphology may be affected by mitochondrial dysfunction. Fiber type abnormalities including varying distribution of type I and II fibers as well as general or selective atrophy have been reported in patients with various mitochondrial respiratory chain dysfunctions (Gallanti *et al.*, 1992; Muranaka *et al.*, 1997; Enns *et al.*, 2005) but not in adults with mitochondrial myopathy. One study including rats with modeled mitochondrial myopathy (Venhoff *et al.*, 2012) directly assessed skeletal muscle fiber type adaptations in response to a primary defect of the mitochondrial respiratory chain and found a transformation from type I to type II fibers. Therefore, a further aim of the present study was to determine whether skeletal muscle morphology is affected in patients with mitochondrial myopathy. We hypothesized that skeletal muscle morphology is altered with mitochondrial myopathy leading to a shift towards more type II fibers.

Materials and methods

Ethical approval

The present experimental protocol was approved by the Cantonal Ethics Committee of Zurich (KEK 2012-0363) and was conducted in accordance with the Declarations of Helsinki. Participants were fully informed about the purposes, benefits and risks associated with this study and gave their written informed consent prior to the initiation of the experiments.

Participants

Six patients with mitochondrial myopathy (one male and five females, 53 ± 9 y), and eight age- and gender-matched healthy controls (two males and six female, 58 ± 14 yr), voluntarily participated in this study. Participants' characteristics are presented in Table 2. Recruited patients (Table 1) were selected from a patient base followed at the neuromuscular research center (Department of Neurology, University Hospital Zurich, Switzerland) and matched by gender, age and as far as possible by physical activity patterns (assessed by interrogation) to healthy controls. In all patients biopsy findings (histochemical succinate dehydrogenase (SDH) staining technique together with electron microscopy and/or molecular genetic analysis of muscle mtDNA) were in accordance with the diagnosis of mitochondrial myopathy. Apart from manifested histological signs of myopathy and/or serum phosphocreatine kinase (CK) elevation, patients featured a history of muscle weakness, exercise intolerance or exercise-dependent myalgia (Table 1). Prior to any experiments, study participants were clinically examined and were screened to exclude potential pregnancy (urine sample) as well as any cardiac and/or respiratory disease. Several metabolic parameters including plasma lactate, glucose, insulin, leptin, glycated hemoglobin (HbA_{1c}), thyroid stimulating hormone (TSH) and CK were measured prio to any study examinations and exercise. Cardiac and respiratory health was assessed by 24 h - electrocardiogram, cardiac ultrasound scan, spirometry tests and laboratory examination (lipid profile, glucose, precursorprotein brain natriuretic peptide (proBNP)). Patients and controls were neither completely sedentary nor highly trained and none of them exhibited diabetes, coronary heart disease, peripheral vascular disease or clinically significant hyperlipidemia.

Experimental design

Clinical and exercise-physiological assessments were performed. Clinical assessments consisted of neurological examination, muscle strength examination, specific health questions (SF-36 Health Survey), Fatigue severity subscale of the Checklist Individual Strength (CIS-fatigue), recorded pain (visual analogue scale, VAS) and a skeletal muscle biopsy obtained from the *vastus lateralis* muscle. Exercise-physiological assessments included a scan for body composition, an incremental exercise test, a constant-load test and a knee extension torque measurement.

Body composition measurement

Total mass, bone mineral content (BMC), fat mass, percentage body fat, as well as total lean mass and lean soft tissue mass of the legs were determined by performing dual-energy X-ray absorptiometry (DXA) measurements using a densitometer (Lunar iDXA^{TM}, GE Healthcare, Madison, WI, USA).

Exercise testing

Each study participant conducted an incremental exercise test on an electrically braked cycle ergometer until volitional exhaustion (Ergoselect 200K, Ergoline, Bitz, Germany) to determine $\dot{V}O_{2peak}$ and P_{peak}. Additionally, all participants performed a constant-load test at 85 % P_{peak} to establish time to exhaustion (t_{lim}). Pulmonary gas exchange and ventilation were continuously recorded during both exercise tests using an online gas collection system (Innocor™ M400, Innovision, Odense, Denmark), where O_2 and CO_2 concentration were continuously measured and monitored as breath-by-breath values. The gas analysers and the flowmeter of the applied spirometer were calibrated prior to and after each test according to the manufacturer's instructions. Throughout all cycling tests, heart rate was recorded (Polar S610i, Polar Electro, Kempele, Finland) and perceived exertion was interrogated by a Borgh Scale. In addition, blood pressure was registered on a regular basis during the incremental cycling test. The incremental cycling test consisted of a 3 min rest phase at 0 W, followed by exercise at 25 W with power increments of 25 W every 120 s until volitional fatigue. Pedal cadences were freely chosen by the participants but had to remain constant throughout the test and lay between 60 and 80 revolutions per minute (rpm). $\dot{V}O_{2peak}$ was determined as the highest mean over a 10 s period. The respiratory exchange ratios (RER) for submaximal power values were absolutely calculated as the mean from 90 - 120 s at 25 W (RER_{25W}) and relatively as the mean over 10 s from the ventilation and power stages according to 50 % P_{peak} (RER_{rel}). The constant-load test included a 3 min rest phase at 0 W,

a 3 min warm-up of 1 min at 40 % and 2 min at 60 % that was followed by the constant power output according to 85 % P_{peak} until volitional exhaustion.

Isokinetic dynamometry

Knee extensor maximal voluntary contraction torque (MVC) was assessed using an isokinetic dynamometer (Con-Trex MJ, Physiomed Elektromedizin, Schnaittach/Laipersdorf, Germany). Participants' bodies were stabilized with straps and handles according to the guidelines of the manufacturer. Each participant performed 3 maximal knee extensions (ω = 3.14 rad s^{-1}) separated by 1 min rest to assess MVC, whereby only the highest value out of the 3 trials was used for statistical analysis.

Skeletal muscle sampling

After a coagulation test, skeletal muscle biopsies were obtained under standardized conditions from the *vastus lateralis* muscle under local anesthesia (1 % lidocaine) of the skin and superficial muscle fascia, using the Bergström technique with a needle modified for suction. The biopsy was immediately dissected macroscopically free of fat and connective tissue and divided into sections for actual measurement of mitochondrial respiratory capacity, later transmission electron microscopy (TEM) and histochemistry. The part of the biopsy for the determination of mitochondrial respiratory capacity was directly placed in ice-cold biopsy preservation solution. For TEM, pieces of around 1 mm^3 of each muscle biopsy were chemically fixed in 2.5 % glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3), stored at room temperature (RT) for 24 h and thereafter at 4 °C until all samples were collected. Muscle tissue for immunohistochemistry was instantly mounted in an embedding medium (Tissue-Tek®, Sakura, Zoeterwoude, The Netherlands), snap frozen in isopentane cooled to −160 °C with liquid nitrogen, and subsequently stored at −80 °C until further processing.

Mitochondrial respiration measurement

Samples were prepared as described in detail previously (Jacobs *et al.*, 2012). Summarised, after mechanical fiber separation, chemical permeabilization in biopsy preservation solution and washing in mitochondrial respiration medium 05, muscle bundles were blotted dry and measured for wet weight (ww) in a balance-controlled scale (XS205 DualRange Analytical Balance, Mettler-Toledo AG, Greifensee, Switzerland). Respiration measurements were subsequently performed in mitochondrial respiration medium 06. O_2 consumption of the individual muscle tissue was thereby measured at 37 °C using the high-resolution Oxygraph-2k (Oroboros, Innsbruck, Austria). Standardised instrumental and chemical calibrations were

performed as recommended by the manufacturer and described previously (Jacobs *et al.*, 2012). O_2 flux was automatically calculated by the software, accounting for nonlinear changes in the negative time derivative of the O_2 concentration signal (DatLab, Oroboros, Innsbruck, Austria). Experiments were performed as duplicates in a hyperoxygenated environment in order to prevent any potential O_2 diffusion limitation. Thereby, O_2 concentration ranged between 200 and 450 nmol mL^{-1} within the chambers.

Respiratory titration protocol

The respiratory measurement protocol was specific to the analysis of individual aspects of respiratory capacity and coupling control efficiency during several substrate states induced via separate titrations. All titrations were added in series as presented, whereby the concentrations of substrates, uncouplers and inhibitors used were based on prior experiments (Jacobs *et al.*, 2012). The titration protocol was modified from previous protocols where they are described in detail (Jacobs *et al.*, 2012). In short, leak respiration in absence of adenylates (L_N) was induced with the addition of octanoyl carnitine (0.2 mM) and malate (2 mM). Specifically, L_N represents the resting O_2 consumption of an unaltered and intact electron transport system (ETS) free of adenylates. Maximal electron flow through electron-transferring flavoprotein and maximal fatty acid oxidative capacity (P_{ETF}) was subsequently determined following the addition of ADP (5 mM). Electron capacity through complex I (P_{CI}) was then induced following the additions of pyruvate (5 mM) and glutamate (10 mM). Maximal oxidative phosphorylation capacity (P) was induced with the addition of succinate (10 mM). P thereby represents respiration that is resultant to saturating concentrations of ADP and substrate supply both for complex I and II. As an internal control for the integrity of the mitochondrial preparation, the mitochondrial outer membrane was assessed with the addition of cytochrome C (10 µM). Following, ATP synthase was inhibited by the titration of oligomycin (1 µM) that lead to oligomycin-induced leak respiration (L_{Omy}). L_{Omy} represents the corresponding leak state to P. In L_{Omy} the chemiosmotic gradient is at maximum because of maximal substrate supply and inhibition of complex V (ATP synthase). Additionally, O_2 flux is at minimum and is representative of proton leak, slip, cation cycling and overall dyscoupling. By uncoupling ATP synthase from the electron transport chain with the step-wise titration (4 x 0.5 µM) of the proton ionophore carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone (FCCP) phosphorylative restraint of electron transport was assessed reaching ETS capacity (E). In order to inhibit CI and to assess electron flow specific to complex II (P_{CII}) rotenone (0.5 µM) was added. P_{CII} is thereby not influenced by the preceding addition of FCCP, which was verified with separate protocols in our laboratory.

The addition of antimycin A (2.5 µM) that inhibits complex III allows the determination and correction of residual O_2 consumption (ROX), which is indicative of non-mitochondrial O_2 consumption in the chamber. Finally, respiration measurements were terminated by simultaneous titration of ascorbate and TMPD to assess complex IV (COX) activity. Ascorbate and TMPD represent redox substrates that donate electrons directly to COX. Correction of O_2 flux for the side reaction of auto-oxidation was conducted by chemical calibration experiments prior to the measurements.

Transmission electron microscopy

The chemically fixed tissue was washed 3 x in 0.1. M cacodylate buffer and consecutively post-fixed in 1 % osmiumtetroxid in 0.1. M cacodylate buffer for 2 h at RT. After 3 x washing with milliQ H_2O samples were block-stained with uranyl acetate (2 % in milliQ H_2O) overnight at RT. The subsequent graded dehydration was conducted in a tissue processor (Leica EM TP, Leica Microsystems, Wetzlar, Germany) according to the following protocol: 10 min 70 % EtOH, 15 min 96 % EtOH, 4 x 30 min 100 % EtOH, 2 x 5 min and 2 x 10 min propylene oxide, 2 x 1 h 1:1 propylene oxide/Epon, 1 h 100 % Epon, 100 % Epon over night, 3 h Epon. Finally, samples were embedded in 100 % Epon in a non-oriented (isotropic) way and Epon was polymerized for 48 h at 60 °C. Ultrathin sections were cut on an ultramicrotome (Ultracut E ultramicrotome, Reichert, NY, USA) with a diamond knife (Diatome, Biel, Switzerland) and placed on 50 mesh hexagonal cupper grids (Plano GmbH, Wetzlar, Germany). Specifically, from each block three ultrathin sections (70 nm) were obtained at two depths separated by 20 µm and placed onto two grids. Finally, the grids were contrasted with lead citrate (Reynolds, 1963). Micrographs were obtained in a FEI Tecnai G2 Spirit electron microscope (Tecnai G2 Spirit, FEI, Hillsboro, OR, USA) mounted with an Orius SC1000 charged-coupled device (CCD) camera (Gatan, Pleasanton, GA, USA) and interfaced with the TEM software (TEM User Interface, FEI, Hillsboro, OR, USA). Micrographs were acquired in a uniform random systematic order. For each grid three predetermined positions were marked for further imaging. At each position an area of 222 x 146 μm^2 (pixel size of 57.7 nm) was used for automated image capturing by the TEM photomontage software. A random starting point was selected for the first micrograph whereafter further micrographs were taken at fixed x-, y-intervals of 64 and 42 µm, respectively. Nine 15.9 x 10.5 μm^2 micrographs (3840 x 2528 pixels) were captured in each area. Consequently, for each biopsy a total of 54 micrographs were acquired (9 micrographs per 3 positions per 2 sections per sample). Subsequently, skeletal muscle volume density of mitochondria ($Mito_{VD}$) was estimated by point counting in combination with Cavalieri's principle (West, 2012) using the Stereo-Investigator software

(MBF Bioscience, Williston, ND, USA). For point counting a grid spacing of 1 µm along bot x- and y-axis was applied. Each point was assigned as either intermyofibrillar (IMF_{VD}) or subsarcolemmal (SS_{VD}) mitochondrial volume density, lipid droplet volume density (LD_{VD}), skeletal muscle or "nothing". SS mitochondria were defined as the mitochondria that were not separated by myofibrils from the sarcolemma.

Histochemistry

Consecutive transverse sections (8 µm) were cut at three depths on a microtome (Leica CM 1850, Leica Biosystems, Wetzlar, Germany) at −22 °C, mounted on glass cover slides (Superfrost Plus, Thermo Fisher Scientific Inc., Rockford, IL, USA), set to air dry and stored at -20 °C until further processing. The serial sections were then fixed in 3 % neutral buffered formalin at RT for 45 min, briefly washed and blocked with 5 % goat serum. Thereafter, the sections were firstly incubated with a primary antibody against myosin heavy chain (MyHC) isoform I (Novocastra Lyophilized Mouse Monoclonal Antibody Myosin Heavy Chain (slow), NCL-MHCs, Leica Biosystems, Wetzlar, Germany) and secondly with goat anti-rabbit IgG secondary antibody conjugated with Alexa Fluor 488 (Thermo Fisher Scientific Inc., Rockford, IL, USA). The sarcolemma of the skeletal muscle fibers were visualized by incubation with a primary antibody against laminin (Novocastra Lyophilized Mouse Monoclonal Antibody Laminin, NCL-LAMININ, Leica Biosystems, Wetzlar, Germany) in combination with goat anti-rabbit IgG secondary antibody, Alexa Fluor 647 conjugate (Thermo Fisher Scientific Inc., Rockford, IL, USA). An automated upright microscope system was used for digitizing the sections (Leica, DM5500 B, Leica Microsystems, Wetzlar, Germany). Skeletal muscle fiber type distribution was determined according to their MyHC-I and MyHC-II isoforms and classified into type I and type II fibers. For all analyses, only fibers fully encircled by adjacent fibers were evaluated using Adobe Photoshop Pro CS6 (Adobe Systems Incorporated, San Jose, CA, USA). Fiber cross-sectional area (CSA) was determined by encircling the boundaries of the muscle cells of at least 50 fibers per fiber type. Only fibers with a circularity higher than 0.7 were considered for analysis (perfect circle = 1.0).

Statistical analysis

All data are presented as mean ± SD in text and figures. The statistical analysis was conducted using the software SPSS Statistics 22.0 (SPSS, Chicago, IL, USA). After verification of normal distribution of the data, two-tailed unpaired samples *t*-tests were conducted to test the null hypothesis stating no difference between patients with

mitochondrial myopathy and health controls. For all statistical analyses, a value of $P < 0.05$ was considered significant. Spearman's rank correlation coeffiencts were calculated to determine potential dependences of several variables. The number of patients and controls included in each data set are always indicated and can vary due to inability of cycling above a power output of 0 W (1 case) or technical problems with the devices (2 cases).

Results

All participants completed the skeletal muscle biopsy procedure and DXA measurement. Except from one patient who was not able to cycle against a certain resistance (25 W), all participants conducted the incremental and the constant-load cycling exercise test. Due to technical reasons, one patient and one control could not perform the isokinetic dynamometry test. There was no difference in age or weight between groups (Table 2).

Body composition

Body composition did not differ between patients and controls in any of the tested parameters (Table 2).

Oxygen kinetics

$\dot{V}O_{2peak}$ was 45 % lower (P < 0.05) in mitochondrial myopathy patients than in healthy controls, with a broad range in both groups (Table 2). In accordance, P_{peak} was 53 % lower (P < 0.05) with mitochondrial myopathy. However, RER at the same absolute and relative power output did not differ between groups, suggesting similar metabolic regulations in patients and controls at submaximal work rates.

Skeletal muscle mitochondrial volume density and respiratory capacity

Maximal mitochondrial respiration specific to each individual mitochondrial complex (mass-specific respiration, respiration per mg ww) was lower (P < 0.05) in patients with mitochondrial myopathy vs. healthy controls (Figure 1A). In particular, difference in mass-specific respiratory capacity with mitochondrial myopathy amounted to 50 (P_{CI}), 46 (P_{CII}), 45 (P), 48 (COX) and 47 % (ETS). Additionally, $Mito_{VD}$ was on average 23 % lower (P < 0.05) in patients, which was related to the lower IMF_{VD} (Table 3). Moreover, after normalizing mitochondrial respiratory capacity to $Mito_{VD}$ all respiratory states specific to the five mitochondrial complexes were lower (P < 0.05, Figure 1B) in patients (28 (P_{CI}), 24 (P_{CII}), 23 (P), 28 (COX) and 24 % (ETS)), suggesting quantitative and qualitative alterations in mitochondrial function with mitochondrial myopathy. In contrast, LCR did not differ (P = 0.501) between groups (mitochondrial myopathy 0.42 ± 0.07; controls 0.44 ± 0.05).

Skeletal muscle fiber type distribution

Mitochondrial myopathy demonstrated a higher (P < 0.001) ratio of FT to ST fibers and a tendency (P = 0.070) towards lower CSA, indicating potential skeletal muscle atrophy (Table 3). However, CSA specific to ST and FT fibers did not differ (P = 0.133; P = 0.127) between patients and controls (Table 3).

Correlations

The mutation load in the 6 patients ranged from 12 to 95 %. There was no relationship between percentage mutation and any of the other determined variables. $\dot{V}O_{2peak}$ was positively correlated (P < 0.05) to P (ρ = 0.626), $Mito_{VD}$ (ρ = 0.577) and P normalized to $Mito_{VD}$ (ρ = 0.648). Moreover, P, $Mito_{VD}$, P/$Mito_{VD}$ as well as lean mass were all related to CSA of ST fibers (ρ = 0.653, ρ = 0.600, ρ = 0.618, ρ = 0.578; P < 0.05).

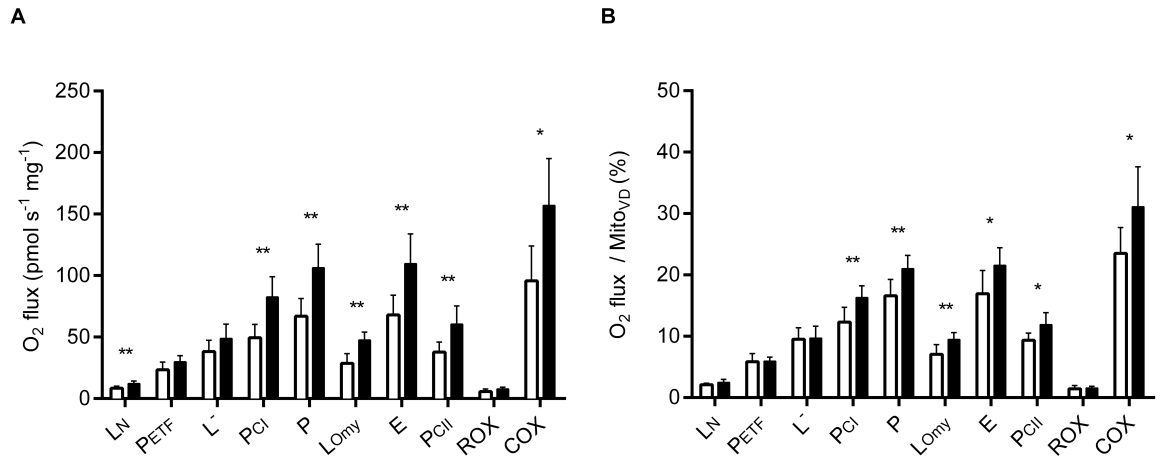

Fig. 1 Mass-specific mitochondrial **(A)** and mitochondrial-specific **(B)** respiratory capacity (normalized to $Mito_{VD}$) in patients with mitochondrial myopathy (white bars) and healthy controls (black bars). L_N, leak respiration without adenylates; P_{ETF}, fatty acid oxidative capacity; P_{CI}, respiratory capacity of complex I; P, oxidative phosphorylation capacity; L_{Omy}, oligomycin-induced leak respiration; E, electron transport system capacity; P_{CII}, respiratory capacity of complex II; ROX, residual oxygen consumption; COX, respiratory capacity of complex IV. Values are mean ± SD. *P < 0.05; **P < 0.01. $n_{Patients}$ = 6, $n_{Controls}$ = 8

Table 1 Clinical and molecular characteristics of patients with mitochondrial myopathy

patient	sex	age of onset (yr)	diagnosis	clinical features	$[CK]_{rest}$ ($U\ L^{-1}$)	$[L^-]_{rest}$ ($mmol\ L^{-1}$)	genetic defect	mutant mtDNA (%)	muscle biopsy
1	f	20	CPEO	ptosis, external ophthalmoplegia, facial weakness, slight general weakness	219	normal	4977 bp	95	COX^-, ragged blue fibers
2	m	33	CPEO	ptosis, external ophthalmoplegia, general weakness, exercise intolerance	401	normal	4405 bp	90	unremarkable
3	f	62	MM	mild proximal lower extremities weakness	normal	normal	5-13 kbp	49-71	COX^-, ragged blue fibers
4	f	12	MM	exercise induced myalgia	206	normal	10-13 kbp	35	COX^-, SDH^+, ragged red fibers
5	f		MM	exercise induced myalgia	normal	normal	6-10 kbp	62	ragged red fibers
6	f	39	MM	exercise induced myalgia	normal	5.3	8-12 kbp	12-36	unremarkable

$[CK]_{rest}$, blood creatine kinase concentration at rest; COX^-, cytochrome oxidase negative fibers; CPEO, chronic progressive external ophthalmoplegia; $[L^-]_{rest}$, blood lactate concentration at rest; MM, mitochondrial myopathy; mtDNA, mitochondrial DNA; SDH^+, succinate dehydrogenase positive fibers

Table 2 Physiological characteristics of patients with mitochondrial myopathy (n = 6) and healthy controls (n = 8)

	patients	controls	*P*-value
age (yr)	53 ± 9	58 ± 14	0.437
weight (kg)	75 ± 18	67 ± 12	0.376
height (cm)	162 ± 7	167 ±7	0.238
BMC (kg)	2.33 ± 0.18	2.36 ± 0.43	0.867
fat mass (kg)	23.1 ± 16.3	14.2 ± 11.6	0.255
lean mass (kg)	38.4 ± 13.3	41.4 ± 13.9	0.683
lean mass legs (kg)	19.3 ± 11.1	19.0 ± 8.8	0.951
total body fat (%)	39 ± 13	31 ± 6	0.153
relative $\dot{V}O_{2peak}$ (mL min^{-1} kg^{-1})	19.8 ± 6.8[a]	32.6 ± 7.3	< 0.01
P_{peak} (W)	78 ± 21[a]	165 ± 60	< 0.05
$[L^-]_{rest}$ (mmol L^{-1})	1.64 ± 0.88	1.11 ± 0.62	0.207
RER_{25W} (-)	0.72 ± 0.06[a]	0.69 ± 0.06	0.447
RER_{rel} (-)	0.78 ± 0.08[a]	0.77 ± 0.05	0.923
HR_{peak}(min^{-1})	144 ± 28[a]	158 ± 16	0.257
t_{lim} (s)	278 ± 154[a]	541 ± 321	0.118
MVC (Nm)	54.3 ± 41.9[a]	80.6 ± 43.3[b]	0.278

Values are represented as means ± SD. $[L^-]_{rest}$, blood lactate concentration at rest; BMC, bone mineral content; HR_{peak}, peak heart rate; MVC, maximum voluntary contraction; P_{peak}, peak power; relative $\dot{V}O_{2peak}$, peak oxygen uptake per kg body weight; RER_{25W}, respiratory exchange ratio at 25 W; RER_{rel}, respiratory exchange ratio at 50% P_{peak}; t_{lim}, time to exhaustion. [a] n = 5; [b] n = 7

Table 3 Skeletal muscle properties of patients with mitochondrial myopathy (n = 6) and healthy controls (n = 8)

	patients	controls	*P*-value
Type I fibers (%)	34.8 ± 3.6	55.7 ± 5.9	< 0.001
Type II fibers (%)	65.2 ± 3.6	44.3 ± 5.9	< 0.001
CSA total (μm^2)	3438 ± 915	4698 ± 1328	0.070
CSA type I fibers (μm^2)	3991 ± 1211	5234 ± 1566	0.133
CSA type II fibers (μm^2)	3178 ± 852	4359 ± 1592	0.127
$Mito_{VD}$ (%)	4.0 ± 0.5	5.1 ± 0.8	< 0.05
IMF_{VD} (%)	3.4 ± 0.4	4.1 ± 0.5	< 0.05
SS_{VD} (%)	0.6 ± 0.3	1.0 ± 0.5	0.118
LD_{VD} (%)	0.4 ± 0.1	0.4 ± 0.3	0.660

Values are represented as means ± SD. CSA, cross sectional area; IMF_{VD}, intermyofibrillar mitochondrial volume density; LD_{VD}, lipid droplet volume density; $Mito_{VD}$, mitochondrial volume density; SS_{VD}, subsarcolemmal mitochondrial volume density

Discussion

The main finding of the present study is that skeletal muscle mitochondrial function in mitochondrial myopathy patients is lower than in healthy controls and that this is the result of a reduced mitochondrial volume density as well as diminished intrinsic mitochondrial function. Furthermore, patients exhibited a shift in skeletal muscle fiber type towards more type II fibers and tended to an atrophic muscle phenotype. Moreover, maximal O_2 uptake and exercise performance in patients with mitochondrial myopathy were lower than in the included healthy controls. Mitochondrial volume density and maximal oxidative phosphorylation capacity were positively correlated to $\dot{V}O_{2peak.}$ Thus, mitochondrial myopathy is associated with decreased mitochondrial quantity and quality and diminished the capacity for whole-body maximal O_2 uptake.

This is the first study determining skeletal muscle mitochondrial volume density and respiratory capacity of each single complex in permeabilized fibers from mitochondrial myopathy patients in comparison to age- and gender-matched healthy controls. Thereby, not only $Mito_{VD}$ but also the mass-specific (per mg ww) and mitochondrial-specific (normalized to $Mito_{VD}$) respiratory capacity of each individual complex were affected by mitochondrial myopathy, indicating an evident impairment of innate mitochondrial function. Together, the lower mitochondrial quantity and quality may limit skeletal muscle's ability to extract O_2 from the blood (Hoppeler *et al.*, 1987) and finally leading to an impaired maximal oxygen uptake capacity of the whole body. Particularly, the lower O_2 extraction capacity of the skeletal muscle is likely to result in an exaggerated systemic O_2 delivery relative to O_2 utilization, i.e. a hyperkinetic circulation, causing typical symptoms of mitochondrial myopathy as metabolic acidosis, exertional dyspnea, exercise intolerance, low exercise performance and consecutively reduced quality of life (Linderholm *et al.*, 1969; Vissing *et al.*, 1996; Taivassalo *et al.*, 2002; Taivassalo *et al.*, 2003; Heinicke *et al.*, 2011). Hence, the present bioenergetics impairments at the cell organelles' level seem to result in abnormal cardiac and ventilatory responses to increased O_2 requirements as in response to physical activity and exercise (Dandurand *et al.*, 1995; Flaherty *et al.*, 2001; Heinicke *et al.*, 2011) and therewith to cause impaired exercise capacity in patients with mitochondrial myopathy. Noteworthy, exercise capacity in patients with mitochondrial myopathy was lower than in healthy matched controls and varied broadly, reflecting the heterogeneous clinical manifestation and severity of this disease. In addition, $\dot{V}O_{2peak}$ correlated with measures of mitochondrial quantity and quality, indicating an association with reduced skeletal muscle O_2 uptake capacity, which as been indicated by previous studies in our laboratory before (Jacobs *et al.*, 2013; Jacobs and

Lundby, 2013). In support, since $\dot{Q}_{peak}$ has been reported not to differ between mitochondrial myopathy and healthy controls (Taivassalo *et al.*, 2003), diminished $\dot{V}O_{2peak}$ may be explained by a lower systemic a-vO_2 diff, which not at last represents a marker for mitochondrial oxidative phosphorylation capacity (Linderholm *et al.*, 1969; Vissing *et al.*, 1996; Taivassalo *et al.*, 2002; Taivassalo *et al.*, 2003).

To our knowledge, this is also the first study demonstrating a previously unreported skeletal muscle fiber type transformation from type I to type II fibers in adult patients with mitochondrial myopathy. This finding contrasts a histochemically described type I fiber predominance as a compensation for impaired energy production and mitochondrial function in children with mitochondrial myopathy (Enns *et al.*, 2005). As type 1 fibers usually contain a higher proportion of mitochondria than type II fibers (Gouspillou *et al.*, 2014), an enhanced ratio of type I to type II fibers could increase the number of mitochondria and lead to a potentially greater capacity for energy production (Enns *et al.*, 2005). In turn, decreased oxidative capacity could also be compensated by a switch from an oxidative to a more glycolytic phenotype in order to partially restore muscle strength and energy production, yet leading to higher lactate production that is characteristic for this disease (Vissing *et al.*, 1996; Jeppesen *et al.*, 2003). Lactate in turn represents an important fuel for oxidative metabolism and has been reported to rather enhance energy production than inducing fatigue in patients with mitochondrial myopathy (Jeppesen *et al.*, 2013). In support of our findings, Venhoff *et al.* (2012) reported a fiber type shift from type I to type II fibers in mitochondrial myopathy modeled rats which was paralleld by adjustments in metabolic profile. The lower $Mito_{VD}$ in patients with mitochondrial myopathy, which was related to lower IMF_{VD}, may thereby reflect the decreased type I to type II fiber ratio. SS_{VD} in the present study did not differ and only half of the mitochondrial myopathy patients demonstrated ragged-red fibers (RRF, Table 1). Together these findings, however, contrast previous reports of SS mitochondrial proliferations and simultaneously occurring RRF with mitochondrial myopathy (Di Mauro *et al.*, 1985; Rollins *et al.*, 2001; Enns *et al.*, 2005). Moreover, mitochondrial myopathy modeled mice demonstrated RRF and increased skeletal muscle mitochondrial volume density, particularly in the SS region, wheras respiratory chain enzyme activities were decreased (Wredenberg *et al.*, 2002). Therefore, the authors suggested the higher mitochondrial mass to partly compensate for the respiratory chain deficiency and the reduced mitochondrial ATP production being not as critical for the pathophysiology of mitochondrial myopathy as previously thought (Wredenberg *et al.*, 2002). Accordingly, recent studies displayed altered Ca^{2+} handling in mitochondrial myopathy (Aydin *et al.*, 2009; Gineste *et al.*, 2015), stimulating the discussion of progressive muscle weakness rather than energy deficiency as potential

dominating pathomechanism (Gineste *et al.*, 2015). Collectively, these partially divergent findings may once more reflect the heterogeneous character of this disease and the possible detrimental effect of physical inactivity in mitochondrial myopathy patients, which in turn emphasizes the need for further studies also on alternate pathophysiological mechanisms.

Discriminating exercise intolerance caused by defective mitochondria and the mutation itself from that of disease associated inactivity and hence physicial deconditioning remains challenging. Exercise intolerance inevitably results in reduced levels of habitual physical activity and causes physical deconditioning that in turn leads to a vicious cycle of further deconditioning and progressive exercise intolerance (Taivassalo and Haller, 2004). In spite of matching patients and controls according to age, gender and, as far as possible, to reported physical activity levels, the lower $\dot{V}O_{2peak}$ and P_{peak} in patients with mitochondrial myopathy could be due to sustained physical inactivity associated with the disease-related exercise intolerance. Thereby, the present lower $Mito_{VD}$ and mitochondrial respiratory capacity in patients with mitochondrial myopathy could indeed also simply be a reflection of this vicious cycle. Disuse or physical inactivity, respectively, have been reported to lead to decreases in mitochondrial volume density and hence mitochondrial function (Gram *et al.*, 2014; Tryon, 2014). In line herewith is the lower IMF_{VD} with mitochondrial myopathy, as IMF mitochondria represent a specialization towards energy production for contractile activity (Ferreira *et al.*, 2010). Reduced contractile activity due to physical inactivity in patients with mitochondrial myopathy could have resulted in lower IMF_{VD} and secondary impaired mitochondrial function. In support, muscle homogenates of mice with mitochondrial myopathy due to a COX deficiency exhibited increased mitochondrial mass, oxidative capacity and exercise performance after an endurance exercise training intervention in comparison to sedentary diseased mice (Wenz *et al.*, 2009). However, although the enhanced physical activity led to a preservation of oxidative capacity of 50 – 60% of wild-type levels in comparison to the drop to 10 – 40 % in sedentary diseased mice in 3 month duration, oxidative capacity still remained reduced compared to wild-type controls. Moreover, mitochondrial COX activity was not affected by the exercise intervention (Wenz *et al.*, 2009). The partially sustained oxidative capacity in muscle homogenates together with the persistent COX defect at the mitochondrial level in exercised diseased mice indicates only increased mitochondrial quantity but not quality with exercise (Wenz *et al.*, 2009). Therefore, lower $Mito_{VD}$ but rather not impaired intrinsic mitochondrial function could be explained by physical inactivity due to exercise intolerance.

Congruently, two weeks of physical inactivity induced by one leg immobilization led to reduced mitochondrial respiratory capacity due to changes in mitochondrial content but not intrinsic mitochondrial function (Gram *et al.*, 2014). In the present study, similar total lean mass and lean mass of the lower limbs in mitochondrial myopathy patients and healthy controls may suggest that the diminished mitochondrial volume density, function and exercise performance are possibly associated with the disease itself rather than with inactivity alone. Additionally, patients with mitochondrial myopathy exhibited comparable MVC and t_{lim} as their healthy controls, which contradict different levels of physical activity. In support, mice with mitochondrial myopathy showed a decrease in ATP levels even with exercise, which was less than in sedentary diseased mice but dropped to 30% of wild-type after 10 month (Wenz *et al.*, 2009). Considering the shift towards more type II fibers and the trend to general fiber atrophy, physical inactivity should still be considered as a potential cause. However, muscle disuse affects mainly type I fiber diameter (Booth and Gollnick, 1983; Zhong *et al.*, 2005), which did not differ between patients and controls. This is further supported by a rat model of mitochondrial myopathy, reporting a predominate type II fiber atrophy (Venhoff *et al.*, 2012). Interestingly, with chronic obstructive pulmonary disease (COPD) muscle hypoxia is, as the case with mitochondrial myopathy, associated with an increased proportion of type II fibers (Hildebrand *et al.*, 1991), a reduced number of mitochondria (Gosker *et al.*, 2007), increased glycolytic enzyme activity and an impaird oxidative capacity (Howald *et al.*, 1990). Conclusively, mechanism related to mitochondrial dysfunction and the disease-causing mutations itself could have induced the present pathologic findings. However, physical inactivity could secondarily have led to further aggraviation of decreased exercise capacity and mitochondrial function. Therefore, the present study cannot clarify whether the findings are only related to the disease per se or in addition to inacitivity.

From a clinical point of view, the diagnosis of mitochondrial cytopathies or mitochondrial myopathy, respectively, remains challenging and requires the combined application of multiple methodologies (Milone and Wong, 2013). Blood samples may exhibit increased resting lactate concentrations and elevated CK levels. However, lactic acidosis is often not present and CK levels may be normal or only mildly increased. Functional muscle testing with the subanaerobic threshold exercise test (SATET) is a very specific measure for mitochondrial pathology (Nashef and Lane, 1989) but many mitochondrial myopathy paitents do not exhibit pathological values. Morphological, biochemical and molecular studies in tissue samples allow a diagnosis by the presence of suggestive histopathological findings such as RRF and ragged-blue fibers, cytochrome C oxidase (COX) depleted fibers, variously

formed mitochondria, abnormal cristae and intracristal crystalloid inclusions and the determination of disease-causing mutations in the mtDNA and/or nDNA (Di Mauro *et al.*, 1985; Milone and Wong, 2013). As demonstrated in two of our 6 patients, these typical histological signs of mitochondrial myopathy can be lacking despite presence of a mitochondrial disease (Milone and Wong, 2013). Molecular genetic analysis of mtDNA in tissues may demonstrate heteroplasmic deletions and point mutations. In addition, nDNA mutations need to be considered. For heteroplasmic mtDNA mutations, the degree of heteroplasmy needs to be quantified, but the pathological threshold is poorly defnied, particularly in mitochondrial cytopathy patients at advanaced age. This heteroplasmy, a canonical criteria for the pathogenitity, is not always given as also pathogenic homoplasmic mutations have been described (Wallace *et al.*, 1988; Di Mauro, 2004).

To provide proper patient management, treatment and genetic counseling, a clear diagnosis of mitochondrial cytopathies is necessary. Our results demonstrate that high-resolution respirometry measurements could represent a promising additional tool for the diagnosis of mitochondrial cytopathies. In vitro measurements as the present high-resolution respirometry allow the estimation of the functionality of specific steps implicated in mitochondrial metabolism by the use of various substrates, uncouplers and inhibitors. Such polarographic measurements of O_2 consumption provide more essential information on innate mitochondrial function, as evaluations not only of isolated mitochondria but also of permeabilized cells are possible (Barrientos *et al.*, 2009). In contrast to the isolation procedure, permeabilization of muscle fibers preserves mitochondrial morphology and integrity and allows the examination of the intact mitochondrial network. However, the definition of normal ranges of complex activites in skeletal muscle remains disputed and difficult. Thus, further studies to eliminate these variations and to validate high-resolution respirometry as a diagnostic measure are needed.

In conclusion, mitochondrial myopathy led to diminished skeletal muscle mitochondrial volume density and respiratory capacity of each individual complex that were related to reduced exercise capacity. The affected mitochondrial quantity and quality could be responsible for the impaired exercise capacity and could further aggravate exercise intolerance. Nonetheless, it cannot be excluded that these findings are simply due to physical inactivity as a cause of the disease-related exercise intolerance. However, the lower mitochondrial volume density was attended by a shift in skeletal muscle phenotype from type I to type II fibers, reflecting a possible compensation for the lower mitochondrial respiratory as well as exercise capacity. Finally, the clearly reduced mitochondrial respiratory capacity in

patients with mitochondrial myopathy compared to age- and gender-matched healthy controls proposes high-resolution respirometry measurements in permeabilized skeletal muscle fibers to be a promising additional diagnostic tool.

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References

Aydin J, Andersson DC, Hanninen SL, Wredenberg A, Tavi P, Park CB*, et al.* Increased mitochondrial Ca2+ and decreased sarcoplasmic reticulum Ca2+ in mitochondrial myopathy. Hum Mol Genet 2009; 18(2): 278-88.

Barrientos A, Fontanesi F, Diaz F. Evaluation of the mitochondrial respiratory chain and oxidative phosphorylation system using polarography and spectrophotometric enzyme assays. Curr Protoc Hum Genet 2009; Chapter 19: Unit19 3.

Booth FW, Gollnick PD. Effects of disuse on the structure and function of skeletal muscle. Med Sci Sports Exerc 1983; 15(5): 415-20.

Cohen BH, Gold DR. Mitochondrial cytopathy in adults: what we know so far. Cleve Clin J Med 2001; 68(7): 625-6, 9-42.

Dandurand RJ, Matthews PM, Arnold DL, Eidelman DH. Mitochondrial disease. Pulmonary function, exercise performance, and blood lactate levels. Chest 1995; 108(1): 182-9.

Di Mauro S. Mitochondrial medicine. Biochim Biophys Acta 2004; 1659(2-3): 107-14.

Di Mauro S. Pathogenesis and treatment of mitochondrial myopathies: recent advances. Acta Myol 2010; 29(2): 333-8.

Di Mauro S, Bonilla E, Zeviani M, Nakagawa M, DeVivo DC. Mitochondrial myopathies. Ann Neurol 1985; 17(6): 521-38.

Enns GM, Hoppel CL, DeArmond SJ, Schelley S, Bass N, Weisiger K*, et al.* Relationship of primary mitochondrial respiratory chain dysfunction to fiber type abnormalities in skeletal muscle. Clin Genet 2005; 68(4): 337-48.

Ferreira R, Vitorino R, Alves RM, Appell HJ, Powers SK, Duarte JA*, et al.* Subsarcolemmal and intermyofibrillar mitochondria proteome differences disclose functional specializations in skeletal muscle. Proteomics 2010; 10(17): 3142-54.

Flaherty KR, Wald J, Weisman IM, Zeballos RJ, Schork MA, Blaivas M*, et al.* Unexplained exertional limitation: characterization of patients with a mitochondrial myopathy. Am J Respir Crit Care Med 2001; 164(3): 425-32.

Gallanti A, Prelle A, Chianese L, Barbieri S, Jann S, Schiaffino S*, et al.* Congenital myopathy with type 2A muscle fiber uniformity and smallness. Neuropediatrics 1992; 23(1): 10-3.

Gineste C, Hernandez A, Ivarsson N, Cheng AJ, Naess K, Wibom R*, et al.* Cyclophilin D, a target for counteracting skeletal muscle dysfunction in mitochondrial myopathy. Hum Mol Genet 2015.

Gosker HR, Hesselink MK, Duimel H, Ward KA, Schols AM. Reduced mitochondrial density in the vastus lateralis muscle of patients with COPD. Eur Respir J 2007; 30(1): 73-9.

Gouspillou G, Sgarioto N, Norris B, Barbat-Artigas S, Aubertin-Leheudre M, Morais JA*, et al.* The relationship between muscle fiber type-specific PGC-1alpha content and mitochondrial content varies between rodent models and humans. PLoS One 2014; 9(8): e103044.

Gram M, Vigelso A, Yokota T, Hansen CN, Helge JW, Hey-Mogensen M*, et al.* Two weeks of one-leg immobilization decreases skeletal muscle respiratory capacity equally in young and elderly men. Exp Gerontol 2014; 58: 269-78.

Hassani A, Horvath R, Chinnery PF. Mitochondrial myopathies: developments in treatment. Curr Opin Neurol 2010; 23(5): 459-65.

Heinicke K, Taivassalo T, Wyrick P, Wood H, Babb TG, Haller RG. Exertional dyspnea in mitochondrial myopathy: clinical features and physiological mechanisms. Am J Physiol Regul Integr Comp Physiol 2011; 301(4): R873-84.

Hildebrand IL, Sylven C, Esbjornsson M, Hellstrom K, Jansson E. Does chronic hypoxaemia induce transformations of fibre types? Acta Physiol Scand 1991; 141(3): 435-9.

Holt IJ, Harding AE, Cooper JM, Schapira AH, Toscano A, Clark JB*, et al.* Mitochondrial myopathies: clinical and biochemical features of 30 patients with major deletions of muscle mitochondrial DNA. Ann Neurol 1989; 26(6): 699-708.

Hoppeler H, Hudlicka O, Uhlmann E. Relationship between mitochondria and oxygen consumption in isolated cat muscles. J Physiol 1987; 385: 661-75.

Howald H, Pette D, Simoneau JA, Uber A, Hoppeler H, Cerretelli P. Effect of chronic hypoxia on muscle enzyme activities. Int J Sports Med 1990; 11 Suppl 1: S10-4.

Iqbal S, Ostojic O, Singh K, Joseph AM, Hood DA. Expression of mitochondrial fission and fusion regulatory proteins in skeletal muscle during chronic use and disuse. Muscle Nerve 2013; 48(6): 963-70.

Jacobs RA, Flück D, Bonne TC, Burgi S, Christensen PM, Toigo M*, et al.* Improvements in exercise performance with high-intensity interval training coincide with an increase in skeletal muscle mitochondrial content and function. J Appl Physiol (1985) 2013; 115(6): 785-93.

Jacobs RA, Lundby C. Mitochondria express enhanced quality as well as quantity in association with aerobic fitness across recreationally active individuals up to elite athletes. J Appl Physiol (1985) 2013; 114(3): 344-50.

Jacobs RA, Siebenmann C, Hug M, Toigo M, Meinild AK, Lundby C. Twenty-eight days at 3454-m altitude diminishes respiratory capacity but enhances efficiency in human skeletal muscle mitochondria. FASEB J 2012; 26(12): 5192-200.

Jeppesen TD, Orngreen MC, Van Hall G, Vissing J. Lactate metabolism during exercise in patients with mitochondrial myopathy. Neuromuscul Disord 2013; 23(8): 629-36.

Jeppesen TD, Schwartz M, Olsen DB, Vissing J. Oxidative capacity correlates with muscle mutation load in mitochondrial myopathy. Ann Neurol 2003; 54(1): 86-92.

Larsson NG, Oldfors A. Mitochondrial myopathies. Acta Physiol Scand 2001; 171(3): 385-93.

Linderholm H, Müller R, Ringqvist T, Sornas R. Hereditary abnormal muscle metabolism with hyperkinetic circulation during exercise. Acta Med Scand 1969; 185(3): 153-66.

Milone M, Wong LJ. Diagnosis of mitochondrial myopathies. Mol Genet Metab 2013; 110(1-2): 35-41.

Moggio M, Colombo I, Peverelli L, Villa L, Xhani R, Testolin S*, et al.* Mitochondrial disease heterogeneity: a prognostic challenge. Acta Myol 2014; 33(2): 86-93.

Morgan-Hughes JA, Schapira AH, Cooper JM, Holt IJ, Harding AE, Clark JB. The molecular pathology of respiratory-chain dysfunction in human mitochondrial myopathies. Biochim Biophys Acta 1990; 1018(2-3): 217-22.

Muranaka H, Osari S, Fujita H, Kimura Y, Goto A, Imoto C*, et al.* Congenital familial myopathy with type 2 fiber hypoplasia and type 1 fiber predominance. Brain Dev 1997; 19(5): 362-5.

Nashef L, Lane RJ. Screening for mitochondrial cytopathies: the sub-anaerobic threshold exercise test (SATET). J Neurol Neurosurg Psychiatry 1989; 52(9): 1090-4.

Picard M, Ritchie D, Wright KJ, Romestaing C, Thomas MM, Rowan SL*, et al.* Mitochondrial functional impairment with aging is exaggerated in isolated mitochondria compared to permeabilized myofibers. Aging Cell 2010; 9(6): 1032-46.

Reynolds ES. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J Cell Biol 1963; 17: 208-12.

Rollins S, Prayson RA, McMahon JT, Cohen BH. Diagnostic yield muscle biopsy in patients with clinical evidence of mitochondrial cytopathy. Am J Clin Pathol 2001; 116(3): 326-30.

Rossignol R, Letellier T, Malgat M, Rocher C, Mazat JP. Tissue variation in the control of oxidative phosphorylation: implication for mitochondrial diseases. Biochem J 2000; 347 Pt 1: 45-53.

Rustin P, Chretien D, Bourgeron T, Gerard B, Rotig A, Saudubray JM*, et al.* Biochemical and molecular investigations in respiratory chain deficiencies. Clin Chim Acta 1994; 228(1): 35-51.

Schapira AH. Mitochondrial disease. Lancet 2006; 368(9529): 70-82.

Schmiedel J, Jackson S, Schafer J, Reichmann H. Mitochondrial cytopathies. J Neurol 2003; 250(3): 267-77.

Taivassalo T, Abbott A, Wyrick P, Haller RG. Venous oxygen levels during aerobic forearm exercise: An index of impaired oxidative metabolism in mitochondrial myopathy. Ann Neurol 2002; 51(1): 38-44.

Taivassalo T, Gardner JL, Taylor RW, Schaefer AM, Newman J, Barron MJ*, et al.* Endurance training and detraining in mitochondrial myopathies due to single large-scale mtDNA deletions. Brain 2006; 129(Pt 12): 3391-401.

Taivassalo T, Haller RG. Implications of exercise training in mtDNA defects--use it or lose it? Biochim Biophys Acta 2004; 1659(2-3): 221-31.

Taivassalo T, Haller RG. Exercise and training in mitochondrial myopathies. Med Sci Sports Exerc 2005; 37(12): 2094-101.

Taivassalo T, Jensen TD, Kennaway N, DiMauro S, Vissing J, Haller RG. The spectrum of exercise tolerance in mitochondrial myopathies: a study of 40 patients. Brain 2003; 126(Pt 2): 413-23.

Tarnopolsky MA, Raha S. Mitochondrial myopathies: diagnosis, exercise intolerance, and treatment options. Med Sci Sports Exerc 2005; 37(12): 2086-93.

Tryon LDV, A.; Memme, J.M.; Crilly, M.J.; Hood D.A. Recent advances in mitochondrial turnover during chronic muscle disuse. Integrative Medicine Research 2014; 3(4): 161-71.

Tuppen HA, Blakely EL, Turnbull DM, Taylor RW. Mitochondrial DNA mutations and human disease. Biochim Biophys Acta 2010; 1797(2): 113-28.

Venhoff N, Lebrecht D, Pfeifer D, Venhoff AC, Bisse E, Kirschner J*, et al.* Muscle-fiber transdifferentiation in an experimental model of respiratory chain myopathy. Arthritis Res Ther 2012; 14(5): R233.

Vissing J, Galbo H, Haller RG. Exercise fuel mobilization in mitochondrial myopathy: a metabolic dilemma. Ann Neurol 1996; 40(4): 655-62.

Wallace DC. Mitochondrial diseases in man and mouse. Science 1999; 283(5407): 1482-8.

Wallace DC, Singh G, Lott MT, Hodge JA, Schurr TG, Lezza AM*, et al.* Mitochondrial DNA mutation associated with Leber's hereditary optic neuropathy. Science 1988; 242(4884): 1427-30.

Wenz T, Diaz F, Hernandez D, Moraes CT. Endurance exercise is protective for mice with mitochondrial myopathy. J Appl Physiol (1985) 2009; 106(5): 1712-9.

West MJ. Estimating volume in biological structures. Cold Spring Harb Protoc 2012; 2012(11): 1129-39.

Wredenberg A, Wibom R, Wilhelmsson H, Graff C, Wiener HH, Burden SJ*, et al.* Increased mitochondrial mass in mitochondrial myopathy mice. Proc Natl Acad Sci U S A 2002; 99(23): 15066-71.

Zhong H, Roy RR, Siengthai B, Edgerton VR. Effects of inactivity on fiber size and myonuclear number in rat soleus muscle. J Appl Physiol (1985) 2005; 99(4): 1494-9.

Mitochondrial function in skeletal muscle of Huntington disease patients

in preparation for submission

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Running title

Skeletal muscle physiology in Huntington disease

Abstract

Purpose. Despite considerable progress in the comprehension of pathomechanisms of Huntington disease (HD) in the brain and central nervous system (CNS), the affection of peripheral tissue as skeletal muscle and possible related pathomechanisms remain elusive. Mitochondrial dysfunction has been proposed to be a major pathogenic factor. Thus, the aim of the present study was to investigate mitochondrial function in skeletal muscle HD patients. **Methods.** Skeletal muscle biopsies were obtained from 10 patients with diagnosed HD (54 ± 7) and 11 age- and gender-matched healthy controls (56 ± 14) to determine skeletal muscle morphology and mitochondrial function. **Results.** Mitochondrial respiratory capacity of complex I (63.9 ± 13.1 vs. 80.6 ± 18.2 pmol O_2 mg^{-1} s^{-1}) and maximal oxidative phosphorylation capacity (88.9 ± 18.7 vs. 106.1 ± 18.4 pmol O_2 mg^{-1} s^{-1}) were impaired with HD ($P < 0.05$) and accompanied by a higher ($P < 0.001$) proportion of type I fibers (67.1 ± 9.2 vs. 39.4 ± 23.3 %). **Conclusion.** HD seems to affect peripheral tissue as skeletal muscle by a shift in fiber phenotype and minor changes in mitochondrial function, potentially supporting mitochondrial dysfunction to be a pathogenic factor.

Key words

Bioenergetics; mitochondria; neuromuscular disease

Abbreviations

BMC, bone mineral content; CK, phosphocreatine kinase; CNS, central nervous system; COX, respiratory capacity of complex IV; CSA, cross-sectional area; DXA, dual-energy X-ray absorptiometry; E, electron transport system capacity; ETS, electron transport system; FCCP, carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone; HD, Huntington disease; L_N, leak respiration without adenylates; L_{Omy}, oligomycin-induced leak respiration; mATPase, myofibrillar adenosinetriphosphatase; P, oxidative phosphorylation capacity; P_{CI}, respiratory capacity of complex I; P_{CII}, respiratory capacity of complex II; P_{ETF}, fatty acid oxidative capacity; ^{31}P-MRS, ^{31}P magnetic resonance spectroscopy; P_{peak}, peak power output; ROX, residual oxygen consumption; UHDRS, Unified Huntington's Disease Rating Scale; $\dot{V}O_{2peak}$, peak oxygen uptake; ww, wet weight.

Introduction

Huntington disease (HD) is a hereditary neurodegenarative disorder characterized by motor disturbance, cognitive loss and psychiatric manifestations (Martin & Gusella, 1986). HD is caused by the expansion of a CAG repeat in exon 1 of the IT-15 gene encoding the protein huntingtin (The Huntington's Disease Collaborative Research Group, 1993). Although the genetic mutation causing HD had been identified, the mechanisms responsible for the pathology have remained widely unclear. As huntingtin was only known to be expressed throughout the brain and cetral nervous system (CNS), for many years attention focused only on the pathologic mechanisms in the brain. However, huntingtin is found also in peripheral tissues including skeletal muscle (The Huntington's Disease Collaborative Research Group, 1993; Sharp *et al.*, 1995). Despite the fact that HD is clinically characterized by a progressive movement disorder which affects motor control and could therewith also deteriorate skeletal muscle function and/or structure, the role of huntingtin and according pathomechanisms still remains elusive. Hence, thorough analysis of skeletal muscle properties offers a promising measure to disclose pathological mechanisms in peripheral tissue and may add to a better understanding of the general pathogenesis of this disorder. Moreover, the elucidation of potential pathologic processes may enhance the development of effective treatment approaches that are lacking so far.

Particularly in the middle or late stages of the disease HD patients often report prominent and problematic weight loss and skeletal muscle wasting (Sanberg *et al.*, 1981; Kirkwood *et al.*, 2001) that can neither be explained by an inadequate caloric intake nor by excessive energy expenditure related to the disease itself (Hamilton *et al.*, 2004; Turner *et al.*, 2007). In addition to this clinical evidence of affected muscle tissue, there have been other signs of skeletal muscle alterations in HD patients but mainly in HD mouse models. Skeletal muscle atrophy that is reported in HD mouse model could be one reason for the weight loss and represents a significant pathological component of HD, also aggravating the movement disorder (Ribchester *et al.*, 2004). The underlying mechanism for this atrophy and whether it is primary or secondary to the disease, however, is unknown. Another potential explanation for the weight loss could be abnormalities of the metabolism, especially impaired mitochondrial function (Lodi *et al.*, 2000; Saft *et al.*, 2005). Some HD patients reported diminished skeletal muscle mitochondrial oxidative metabolism (Lodi *et al.*, 2000; Saft *et al.*, 2005) which supports the hypothesis of mitochondrial dysfunction as a potential factor involved in the pathogenesis of this disease. Collectively, studies on the effect of HD on peripheral tissue as skeletal muscle, the underlying mechanisms as well as its consequences

especially in humans are lacking. Therefore, we aimed to investigate potential alterations in skeletal muscle morphology and mitochondrial function of patients with HD to test the hypothesis that HD also affects and manifests in peripheral tissue. The present findings could offer new insights into the disease mechanism and thereby support the establishment of new therapeutic approaches.

Methods

Ethical approval

The present experimental protocol was approved by the Cantonal Ethics Committee of Zurich (KEK-Nr. 2009-0119) and was conducted in accordance with the Declarations of Helsinki. Participants were fully informed about the purposes, benefits and risks associated with this study and gave their written informed consent prior to the initiation of the experiments. Data of healthy controls were collected from two other studies of our laboratory investigating mitochondrial function in healthy individuals that however have not been published so far.

Participants

A total of 10 patients with the genetically verified diagnosis of HD (6 males and 4 females, 54 ± 7 y), and 11 age- and gender-matched healthy controls (7 males and 4 females, 56 ± 14 y), voluntarily participated in this study. Participants' characteristics are presented in Table 2. Recruited patients (Table 1) were selected from a patient base followed at the neuromuscular research center (Department of Neurology, University Hospital Zurich) and matched by gender, age and as far as possible by physical activity patterns to healthy controls. Prior to any experiments, study participants were clinically examined and were screened to exclude any cardiac, respiratory or metabolic disease and/or any orthopedic pathologies. Serum phosphocreatine kinase (CK) levels needed to be lower than 300 U L^{-1} and motor and cognitive skills of patients needed to be well enough to give written informed consent and to conduct a cycling exercise test (Table 1). Patients and controls were neither completely sedentary nor highly trained and none of them exhibited diabetes, coronary heart disease, peripheral vascular disease or clinically significant hyperlipidemia.

Experimental design

At baseline, clinical and exercise-physiological assessments were performed. Clinical assessments consisted of neurological examination, muscle strength examination, specific health questions and a skeletal muscle biopsy obtained from the *m. vastus lateralis*. Exercise-physiological assessments included a scan for body composition and an incremental exercise test for the determination of exercise capacity.

Body composition measurement

Total mass, bone mineral content (BMC), fat mass, percentage body fat, as well as total and lean soft tissue mass of the leg were determined by performing dual-energy X-ray absorptiometry (DXA) measurements using a densitometer (Lunar iDXA^{TM}, GE Healthcare, Madison, WI, USA).

Exercise testing

Each study participant conducted an incremental exercise test on an electrically braked cycle ergometer until volitional exhaustion (Ergoselect 200K, Ergoline, Bitz, Germany) to determine peak O_2 uptake ($\dot{V}O_{2peak}$) and peak power output (P_{peak}). Pulmonary gas exchange and ventilation were continuously recorded during both exercise tests using an online gas collection system (Innocor™ M400, Innovision, Odense, Denmark), where O_2 and CO_2 concentration were continuously measured and monitored as breath-by-breath values. The gas analysers and the flowmeter of the applied spirometer were calibrated prior to and after each test according to the manufacturer's instructions. Throughout all cycling tests, heart rate was recorded (Polar S610i, Polar Electro, Kempele, Finland) and perceived exertion was interrogated by a Borgh Scale. The incremental cycling test consisted of a 3 min rest phase at 0 W, followed by exercise at 25 W with power increments of 25 W every 120 s until volitional fatigue. Pedal cadences were freely chosen by the participants but had to remain constant throughout the test and lay between 60 and 80 revolutions per minute (rpm). $\dot{V}O_{2peak}$ was determined as the highest mean over a 10 s period.

Skeletal muscle sampling

After a coagulation test, skeletal muscle biopsies were obtained under standardized conditions from the *m. vastus lateralis* under local anesthesia (1 % lidocaine) of the skin and superficial muscle fascia, using the Bergström technique with a needle modified for suction. The biopsy was immediately dissected macroscopically free of fat and connective tissue and divided into sections for actual measurement of mitochondrial respiratory capacity and later histochemistry. The part of the biopsy for the determination of mitochondrial respiratory capacity was directly placed in ice-cold biopsy preservation solution. Muscle tissue for immunohistochemistry was instantly mounted in an embedding medium (Tissue-Tek®, Sakura, Zoeterwoude, The Netherlands), snap frozen in isopentane cooled to −160 °C with liquid nitrogen, and subsequently stored at −80 °C until further processing.

Mitochondrial respiration measurement

Samples were prepared as described in detail previously (Jacobs *et al.*, 2012). Summarised, after mechanical fiber separation, chemical permeabilization in biopsy preservation solution and washing in mitochondrial respiration medium 05, muscle bundles were blotted dry and measured for wet weight (ww) in a balance-controlled scale (XS205 DualRange Analytical Balance, Mettler-Toledo AG, Greifensee, Switzerland). Respiration measurements were subsequently performed in mitochondrial respiration medium 06. O_2 consumption of the individual muscle tissue was thereby measured at 37 °C using the high-resolution Oxygraph-2k (Oroboros, Innsbruck, Austria). Standardised instrumental and chemical calibrations were performed as recommended by the manufacturer and described previously (Jacobs *et al.*, 2012). O_2 flux was automatically calculated by the software, accounting for nonlinear changes in the negative time derivative of the O_2 concentration signal (DatLab, Oroboros, Innsbruck, Austria). Experiments were performed as duplicates in a hyperoxygenated environment in order to prevent any potential O_2 diffusion limitation. Thereby, O_2 concentration ranged between 200 and 450 nmol mL^{-1} within the chambers.

Respiratory titration protocol

The respiratory measurement protocol was specific to the analysis of individual aspects of respiratory capacity and coupling control efficiency during several substrate states induced via separate titrations. All titrations were added in series as presented, whereby the concentrations of substrates, uncouplers and inhibitors used were based on prior experiments (Jacobs *et al.*, 2012). The titration protocol was modified from previous protocols where they are described in detail (Jacobs *et al.*, 2012). In short, leak respiration in absence of adenylates (L_N) was induced with the addition of octanoyl carnitine (0.2 mM) and malate (2 mM). Specifically, L_N represents the resting O_2 consumption of an unaltered and intact electron transport system (ETS) free of adenylates. Maximal electron flow through electron-transferring flavoprotein and maximal fatty acid oxidative capacity (P_{ETF}) was subsequently determined following the addition of ADP (5 mM). Electron capacity through complex I (P_{CI}) was then induced following the additions of pyruvate (5 mM) and glutamate (10 mM). Maximal oxidative phosphorylation capacity (P) was induced with the addition of succinate (10 mM). P thereby represents respiration that is resultant to saturating concentrations of ADP and substrate supply both for complex I and II. As an internal control for the integrity of the mitochondrial preparation, the mitochondrial outer membrane was assessed with the addition of cytochrome C (10 µM). Following, ATP synthase was inhibited by the titration of oligomycin (1 µM) that lead to oligomycin-induced leak respiration (L_{Omy}).

L_{Omy} represents the corresponding leak state to P. In L_{Omy} the chemiosmotic gradient is at maximum because of maximal substrate supply and inhibition of complex V (ATP synthase). Additionally, O_2 flux is at minimum and is representative of proton leak, slip, cation cycling and overall dyscoupling. By uncoupling ATP synthase from the electron transport chain with the step-wise titration (4 x 0.5 µM) of the proton ionophore carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone (FCCP) phosphorylative restraint of electron transport was assessed reaching ETS capacity (E). In order to inhibit CI and to assess electron flow specific to complex II (P_{CII}) rotenone (0.5 µM) was added. P_{CII} is thereby not influenced by the preceding addition of FCCP, which was verified with separate protocols in our laboratory. The addition of antimycin A (2.5 µM) that inhibits complex III allows the determination and correction of residual O_2 consumption (ROX), which is indicative of non-mitochondrial O_2 consumption in the chamber. Finally, respiration measurements were terminated by simultaneous titration of ascorbate and TMPD to assess complex IV (COX) activity. Ascorbate and TMPD represent redox substrates that donate electrons directly to COX. Correction of O_2 flux for the side reaction of auto-oxidation was conducted by chemical calibration experiments prior to the measurements. All respiratory states were expressed per mg ww (mass-specific respiratory capacity) and normalized to COX (mitochondrial-specific respiratory capacity), a biomarker of mitochondrial content (Larsen *et al.*, 2012; Jacobs *et al.*, 2013).

Histochemistry

Consecutive 8 µm sections were cut on a microtome at −25 °C and mounted on glass cover slides for further histochemical analyses. The serial cryocut-cross-sections were stained using the myofibrillar adenosinetriphosphatase (mATPase) method after acid (pH 4.6preincubation according to Item *et al.* (2011). For all analyses, only fibers fully encircled by adjacent fibers were evaluated using Adobe Photoshop Pro CS6 (Adobe Systems Incorporated, San Jose, CA, USA) and were classified according to their isoform into type I and type II fibers. Fiber cross-sectional area (CSA) was determined by encircling the boundaries of the muscle cells of each fiber type. Only fibers with a circularity higher than 0.7 were considered for analysis (perfect circle = 1.0).

Statistics

All data are presented as mean ± SD in text and figures. For all statistical analyses, a value of $P < 0.05$ was considered significant. The statistical analysis was conducted using the software SPSS Statistics 22.0 (SPSS, Chicago, IL, USA). After verification of normal

distribution of the data, unpaired samples *t*-tests were conducted to test the null hypothesis stating no difference between patients with HD and health controls. When data was not normally distributed, a Wilcoxon-Mann-Whitney-U test was performed instead. Spearman's rank correlation coefficients were calculated to determine potential dependences of several variables. The number of patients and controls included in each data set are always indicated and can vary due to technical difficulties.

Results

All participants completed the skeletal muscle biopsy procedure, the DXA measurement and the incremental cycling exercise test. There was no difference in age or weight between groups (Table 2), indicating appropriate matching of patients and participants.

Oxygen kinetics and body composition

$\dot{V}O_{2peak}$ was similar in patients with HD vs. healthy controls (Table 2). In accordance, P_{peak} did not differ (P = 0.219) between groups (Table 2). Moreover, there were no differences in body composition between patients and controls in any of the tested parameters (Table 2). Collectively, these findings suggest similar fitness level of patients and controls.

Skeletal muscle fiber type distribution

There was a clear transition in skeletal muscle fiber type from type II to type I fibers in patients with HD compared to healthy controls (Table 2). In contrast, there was no difference in CSA of any fiber type between patients and controls (Table 2), i.e. no atrophic sign in patients with HD was present.

Skeletal muscle mitochondrial function and density

Mass-specific maximal mitochondrial respiration specific to complex I and oxidative phoshorylative capacity (per mg ww) were 12 % and 9 % lower (P < 0.05) in patients with HD vs. healthy controls (Figure 1A). However, after normalizing mitochondrial respiratory capacity to COX activity, a biomarker of mitochondrial content (Larsen *et al.*, 2012; Jacobs *et al.*, 2013), all respiratory states specific to the five mitochondrial complexes were similar between groups (Figure 1B). Moreover, COX activity did not differ between groups, indicating similar mitochondrial content.

Relations

There was no correlation between UHDRS and $\dot{V}O_{2peak}$ (ρ = −0.233; P = 0.546) or P_{peak} (ρ = −0.218; P = 0.574). Simliarly, UDHRS did not correlate with P_{CI} or P (ρ = 0.200; P = 0.606 or ρ = −0.033; P = 0.932). Furthermore, there was no correlation between fiber type distribution and UDHRS (ρ = 0.200; P = 0.606). However, UDHRS was negatively correlated (P < 0.05) to CSA of type II fibers (ρ = −0.886).

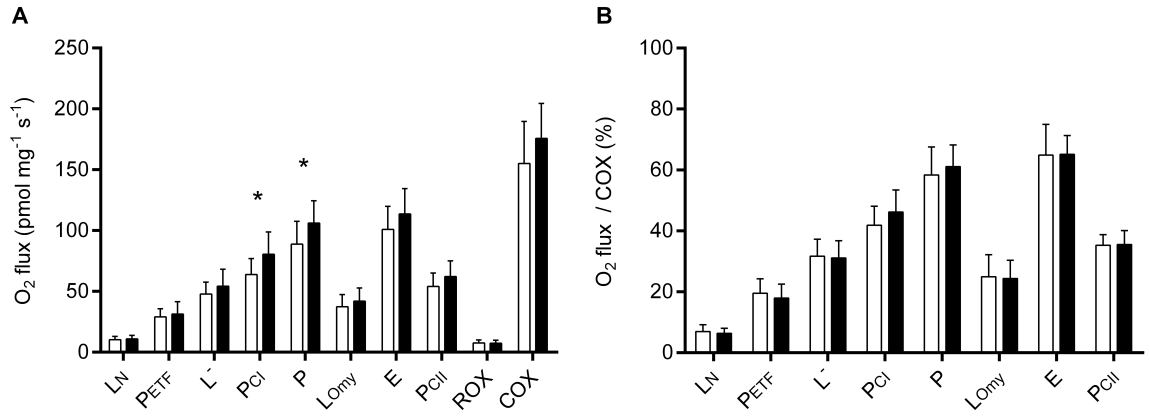

Fig 1. (A) Mass-specific mitochondrial respiratory capacity and **(B)** mitochondrial-specific respiratory capacity (normalized to COX) in patients with Huntington disease (HD, white bars) and healthy controls (black bars). L_N, leak respiration without adenylates; P_{ETF}, fatty acid oxidative capacity; P_{CI}, respiratory capacity of complex I; P, oxidative phosphorylation capacity; L_{Omy}, oligomycin-induced leak respiration; E, electron transport system capacity; P_{CII}, respiratory capacity of complex II; ROX, residual oxygen consumption; COX, respiratory capacity of complex IV. Values are mean ± SD. *P < 0.05; $n_{Patients}$ = 10, $n_{Control}$ = 11

Table 1 Clinical and genetic characteristics of patients with Huntington disease

patient	sex	age of onset (y)	disease duration (y)	UHDRS	CAG repeats
EHD 22	m	52	-	3	39
EHD 24	m	40	20	37	41
EHD 26	m	52	7	20	42
EHD 27	m	37	5	7	43
EHD 28	f	59	-	43	42
EHD 29	f	53	-	6	38
EHD 30	m	-	-	24	43
EHD 31	f	-	-	1	38
EHD 32	m	48	3	11	45
EHD 33	f	56	6	28	41

CAG repeats, cytosine-adenosine-guanosine repeats; UHDRS, Unified Huntington's Disease Rating Scale

Table 2 Physiological characteristics of patients with Huntington disease (n = 10) and healthy controls (n = 11)

	Patients	controls	*P*-value
age (y)	54 ± 7	56 ± 14	0.674
weight (kg)	77 ± 16	74 ± 12	0.635
height (cm)	172 ± 8	173 ±7	0.719
BMC (kg)	2.8 ± 0.5	2.8 ± 0.4	0.881
fat mass (kg)	22.4 ± 8.4	18.2 ± 10.0	0.316
lean mass (kg)	53.3 ± 11.5	47.8 ± 12.9	0.317
lean mass legs (kg)	18.4 ± 4.3	20.0 ± 6.8	0.535
total body fat (%)	29.4 ± 8.1	29.3 ± 5.2	0.973
relative $\dot{V}O_{2peak}$ (mL min^{-1} kg^{-1})	31.0 ± 6.6	34.6 ± 6.5	0.232
P_{peak} (W)	155 ± 50	182 ± 46	0.219
Type I fibers (%)	67.1 ± 9.2	39.4 ± 23.3[a]	< 0.05
Type II fibers (%)	32.9 ± 9.2	60.6 ± 23.3[a]	< 0.05
CSA type I fibers (μm^2)	4134 ± 581[b]	5191 ± 1549[a]	0.193
CSA type II fibers (μm^2)	3652 ± 923[b]	4596 ± 1403[a]	0.193

Values are represented as means ± SD. BMC, bone mineral content; CSA, cross-sectional area; P_{peak}, peak power; relative $\dot{V}O_{2peak}$, peak oxygen uptake per kg body weight. [a] n = 10, [b] n = 7

Discussion

The main finding of this study is a shift from type II fibers to type I fibers without atrophic signs in any of the fiber types. In line therewith, neither body composition nor exercise capacity differed between patients with HD and healthy controls. Mitochondrial respiratory capacity specific to complex I and maximal oxidative phosphorylation capacity per mg ww of tissue were slightly lower in patients with HD. However, when respiratory capacity was normalized to mitochondrial content, the difference disappeared. Despite higher type I fiber distribution, there was no indication for higher mitochondrial content with HD.

The shift in skeletal muscle phenotype to more type I fibers could represent a compensation for the lower respiratory capacity of complex I and oxidative phosphorylation capacity, allowing the maintenance of exercise capacity. In the present study, mass-specific complex I respiratory capacity and maximal oxidative phosphorylation capacity were lower in patients with HD. Noteworthy, this is the first study that determines mitochondrial function in patients with HD by high-resolution respirometry, which allows the preservation of innate mitochondrial function in contrast to other previously applied methods (Lanza *et al.*, 2011; Picard *et al.*, 2011). This complex I deficiency has been previously described in three of four muscle biospies from symptomatic HD patients that showed no further muscle symptoms nor muscle wasting (Arenas *et al.*, 1998). In contrast, mitochondrial content, represented by COX activity (Larsen *et al.*, 2012; Jacobs *et al.*, 2013), did not differ between HD patients and healthy controls and when mitochondrial respiratory capacity was normalized to it, there was no difference in the innate function of any of the mitochondrial complexes. These findings are also in line with previous findings (Turner *et al.*, 2007; Ciammola *et al.*, 2011) that found no difference in respiratory capacity specific to complex I to IV. However, when correlated to disease progression, they found a reduction in complex II/III activity, suggesting lower mitochondrial oxidative capacity with the course of the disease. Additionally, impaired mitochondrial function assessed by ^{31}P magnetic resonance spectroscopy (^{31}P-MRS) in muscle of HD patients (Koroshetz *et al.*, 1997; Lodi *et al.*, 2000; Saft *et al.*, 2005) support the hypothesis that impaired mitochondrial function is involved in the pathogenesis of HD and could contribute to worsening of several clinical parameters as progressive neurodegeneration in HD. The pathophysiological mechanisms that could cause mitochondrial impairment remain speculative. It has been suggested (Arenas *et al.*, 1998) that patients with large CAG repeats have elevated muscle mitochondrial DNA damage that could secondarily lead to mitochondrial respiratory chain defects. Further studies are however needed to strengthen these primary results.

Patients with HD disclosed no difference in weight or in body composition compared to age- and gender-matched healthy controls. These findings are in contrast with earlier reports of severe weight loss and skeletal muscle wasting, especially in the middle and late stages of this disease (Sanberg *et al.*, 1981; Kirkwood *et al.*, 2001). However, in support of our findings, more recent investigation on greater patient cohorts could not find a reduction in weight (Hamilton *et al.*, 2004) or in body composition with HD (Pratley *et al.*, 2000). Hence, weight loss seems not be a consistent feature of HD. Preserved lean mass (Table 2) and unaltered CSA of both fiber types further suggest that HD does not per se lead to skeletal muscle atrophy. However, as there was a correlation between UHDRS and CSA of type II and in consideration of previous studies (Kirkwood *et al.*, 2001; Hamilton *et al.*, 2004) onset of skeletal muscle atrophy with further disease progression in these patients cannot be excluded.

Furthermore, the unchanged body composition was reflected by the comparable exercise capacity in patients and controls, which at first glance seems rather surprising. However, total exercise capacity has previously been shown to be normal in HD patients despite reduced P_{peak} and $\dot{V}O_{2peak}$ in symptomatic HD patients compared to healthy controls (Ciammola *et al.*, 2011). Reasons for this minimal discrepancy to the present study could be lifestyle, as the present study participants overall reported a higher $\dot{V}O_{2peak}$ and therefore were most likely more physically active than the study cohort of Ciammola *et al.* (2011). Usually, HD patients engage in less voluntary physical activity than healthy individuals due to the symptoms of their disease. Nonetheless, their daily free-living energy expenditure is similar to healthy controls because of a higher sedentary energy expenditure (Pratley *et al.*, 2000). It is suggested, that the resting energy expenditure is increased according to the choreaic movement disorder (Pratley *et al.*, 2000). Moreover, chorea could lead to a similar skeletal muscle phenotype as endurance training (Strand *et al.*, 2005). Hence, the choreaic movement disorder of HD patients could also be an explanation for the shift in muscle fiber type from type II to type I fibers reported in this study. Chronic low frequency stimulation of muscle contraction leasds to a transition from fast to slow fibers (Buller *et al.*, 1960; Eken & Gundersen, 1988), indicating changes in electrical activity to be responsible for a switch in skeletal muscle phenotype (Berchtold *et al.*, 2000; Flück & Hoppeler, 2003). Therefore, the transition in fiber type distribution towards more type I fibers with HD could be explained by an aberrant input from CNS manifesting in choreaic movement (Strand *et al.*, 2005), which was however not tested in this study and remains to be investigated.

Conclusion

This is the first study that investigates potential alterations in human skeletal muscle morphology and mitochondrial respiratory capacity of each individual complex in HD patients. HD resulted in a shift from type II fibers to type I fibers without atrophic signs in any of the fiber types. This transition in fiber type was accompanied by lower mitochondrial respiratory capacity specific to complex I and maximal oxidative phosphorylation capacity per mg ww of tissue. However, there was no difference in mitochondrial content with HD. In conclusion, skeletal muscle seems to be affected in patients with HD with a shift in skeletal muscle phenotype and minor mitochondrial alterations. Further studies investigating the underlying mechanism remain required.

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Conflict of interest

The authors declare that they have no conflict of interest.

References

Arenas J, Campos Y, Ribacoba R, Martin MA, Rubio JC, Ablanedo P & Cabello A (1998). Complex I defect in muscle from patients with Huntington's disease. *Ann Neurol* **43,** 397-400.

Berchtold MW, Brinkmeier H & Muntener M (2000). Calcium ion in skeletal muscle: its crucial role for muscle function, plasticity, and disease. *Physiol Rev* **80,** 1215-1265.

Buller AJ, Eccles JC & Eccles RM (1960). Interactions between motoneurones and muscles in respect of the characteristic speeds of their responses. *J Physiol* **150,** 417-439.

Ciammola A, Sassone J, Sciacco M, Mencacci NE, Ripolone M, Bizzi C, Colciago C, Moggio M, Parati G, Silani V & Malfatto G (2011). Low anaerobic threshold and increased skeletal muscle lactate production in subjects with Huntington's disease. *Mov Disord* **26,** 130-137.

Eken T & Gundersen K (1988). Electrical stimulation resembling normal motor-unit activity: effects on denervated fast and slow rat muscles. *J Physiol* **402,** 651-669.

Flück M & Hoppeler H (2003). Molecular basis of skeletal muscle plasticity--from gene to form and function. *Rev Physiol Biochem Pharmacol* **146,** 159-216.

Group THsDCR (1993). A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. The Huntington's Disease Collaborative Research Group. *Cell* **72,** 971-983.

Hamilton JM, Wolfson T, Peavy GM, Jacobson MW, Corey-Bloom J & Huntington Study G (2004). Rate and correlates of weight change in Huntington's disease. *J Neurol Neurosurg Psychiatry* **75,** 209-212.

Item F, Denkinger J, Fontana P, Weber M, Boutellier U & Toigo M (2011). Combined effects of whole-body vibration, resistance exercise, and vascular occlusion on skeletal muscle and performance. *Int J Sports Med* **32,** 781-787.

Jacobs RA, Flück D, Bonne TC, Burgi S, Christensen PM, Toigo M & Lundby C (2013). Improvements in exercise performance with high-intensity interval training coincide with an increase in skeletal muscle mitochondrial content and function. *J Appl Physiol (1985)* **115,** 785-793.

Jacobs RA, Siebenmann C, Hug M, Toigo M, Meinild AK & Lundby C (2012). Twenty-eight days at 3454-m altitude diminishes respiratory capacity but enhances efficiency in human skeletal muscle mitochondria. *FASEB J* **26,** 5192-5200.

Kirkwood SC, Su JL, Conneally P & Foroud T (2001). Progression of symptoms in the early and middle stages of Huntington disease. *Arch Neurol* **58,** 273-278.

Koroshetz WJ, Jenkins BG, Rosen BR & Beal MF (1997). Energy metabolism defects in Huntington's disease and effects of coenzyme Q10. *Ann Neurol* **41,** 160-165.

Lanza IR, Bhagra S, Nair KS & Port JD (2011). Measurement of human skeletal muscle oxidative capacity by 31P-MR spectroscopy: a cross-validation with in vitro measurements. *J Magn Reson Imaging* **34,** 1143-1150.

Larsen S, Nielsen J, Hansen CN, Nielsen LB, Wibrand F, Stride N, Schroder HD, Boushel R, Helge JW, Dela F & Hey-Mogensen M (2012). Biomarkers of mitochondrial content in skeletal muscle of healthy young human subjects. *J Physiol* **590,** 3349-3360.

Lodi R, Schapira AH, Manners D, Styles P, Wood NW, Taylor DJ & Warner TT (2000). Abnormal in vivo skeletal muscle energy metabolism in Huntington's disease and dentatorubropallidoluysian atrophy. *Ann Neurol* **48,** 72-76.

Martin JB & Gusella JF (1986). Huntington's disease. Pathogenesis and management. *N Engl J Med* **315,** 1267-1276.

Picard M, Taivassalo T, Gouspillou G & Hepple RT (2011). Mitochondria: isolation, structure and function. *J Physiol* **589,** 4413-4421.

Pratley RE, Salbe AD, Ravussin E & Caviness JN (2000). Higher sedentary energy expenditure in patients with Huntington's disease. *Ann Neurol* **47,** 64-70.

Ribchester RR, Thomson D, Wood NI, Hinks T, Gillingwater TH, Wishart TM, Court FA & Morton AJ (2004). Progressive abnormalities in skeletal muscle and neuromuscular junctions of transgenic mice expressing the Huntington's disease mutation. *Eur J Neurosci* **20,** 3092-3114.

Saft C, Zange J, Andrich J, Müller K, Lindenberg K, Landwehrmeyer B, Vorgerd M, Kraus PH, Przuntek H & Schols L (2005). Mitochondrial impairment in patients and asymptomatic mutation carriers of Huntington's disease. *Mov Disord* **20,** 674-679.

Sanberg PR, Fibiger HC & Mark RF (1981). Body weight and dietary factors in Huntington's disease patients compared with matched controls. *Med J Aust* **1,** 407-409.

Sharp AH, Loev SJ, Schilling G, Li SH, Li XJ, Bao J, Wagster MV, Kotzuk JA, Steiner JP, Lo A & et al. (1995). Widespread expression of Huntington's disease gene (IT15) protein product. *Neuron* **14,** 1065-1074.

Strand AD, Aragaki AK, Shaw D, Bird T, Holton J, Turner C, Tapscott SJ, Tabrizi SJ, Schapira AH, Kooperberg C & Olson JM (2005). Gene expression in Huntington's disease skeletal muscle: a potential biomarker. *Hum Mol Genet* **14,** 1863-1876.

Turner C, Cooper JM & Schapira AH (2007). Clinical correlates of mitochondrial function in Huntington's disease muscle. *Mov Disord* **22,** 1715-1721.

3. Discussion and Outlook

The aim of the present dissertation was to augment the understanding of skeletal muscle mitochondrial function and to contribute to a comprehensive conception of these organelles. Accordingly, skeletal muscle mitochondrial function and its relation to different other muscle physiological aspects were determined in healthy young and old fitness-level-matched individuals as well as in patients with MM and HD. As the results are discussed in detail in the corresponding manuscripts, the following section provides a summary of the key conclusions, listed separately for each study of this dissertation. The last subchapter will highlight the general conclusion and present an outlook for future research.

3.1 Exercise efficiency is higher in old compared to young men at similar fitness level

This study expands previous research in the field of aging and efficiency by accounting for fitness level of the young and old participants. The findings demonstrate higher EE with advancing age, indicating that impairments formerly related to age were biased by the drawbacks of physical inactivity accompanying a sedentary lifestyle. Additionally, mitochondrial fatty acid oxidative capacity was higher in old individuals and positively correlated to EE, suggesting that older individuals may rely more on fatty acid oxidation and are therefore more efficient. The higher capacity of fatty acid oxidation was further reflected by a lower respiratory exchange ratio with age. In contrast to our hypothesis, neither fiber type distribution nor mitochondrial content differed between old and young individuals. Similarly, maximal oxidative phosphorylation did not differ with age and was not related to EE. However, a potentially higher recruitment of ST fibers mirrored by a tendency towards lower FT fiber cross-sectional area in old participants could partly explain the higher reliance on fatty acid oxidation and thereby the higher EE with increasing age. Further research investigating additional influencing factors on EE and the interdependency of age is needed, as EE represents a key determinant of exercise capacity and thus mobility of the elderly.

3.2 Patients with mitochondrial myopathy exhibit diminished mitochondrial quantity and quality and a shift in skeletal muscle phenotype

This study demonstrates that both mitochondrial volume density and intrinsic mitochondrial function were affected in patients with MM. In particular, maximal respiratory capacity of each single complex was lower with MM. Additionally, MM led to a shift in skeletal muscle phenotype towards more type II fibers and a tendency to general muscle fiber atrophy. Furthermore, $\dot{V}O_{2peak}$ in patients with MM was reduced and correlated to mitochondrial volume density and maximal oxidative phosphorylation capacity. Hence, the affected mitochondrial quantity and quality might be responsible for the impaired exercise capacity and could thereby aggravate exercise intolerance. Nevertheless, this study could not clarify whether the disease per se led to mitochondrial impairments. Physical inactivity as a consequence of the disease-related exercise intolerance could as well have at least partly contributed to lower mitochondrial content and function with MM. Collectively, MM-related dysfunctions further promote exercise intolerance, which in turn seems to be a fundamental consequence of a defective respiratory chain due to pathogenic mutations of skeletal muscle mtDNA and/or nDNA.

3.3 Mitochondrial function in skeletal muscle of Huntington disease patients

This study extends previous investigations on potential alterations in peripheral tissue with HD by the examination of human skeletal muscle morphology and mitochondrial function specific to each separate complex in patients with HD. The main findings were a shift from type II fibers to type I fibers without atrophic signs in any of the fiber types. This translation in skeletal muscle phenotype with HD coincided with a lower mitochondrial respiratory capacity specific to complex I and maximal oxidative phosphorylation capacity per mg wet weight of tissue. However, despite higher type I fiber proportion in patients with HD, mitochondrial content did not differ. Additionally, when respiratory capacity was normalized to mitochondrial content there was no difference between groups. Collectively, the present findings indicate that skeletal muscle is affected in patients with HD, at least by a shift in skeletal muscle phenotype and minor mitochondrial alterations. Nevertheless, further research on the underlying mechanisms is required.

3.4 Conclusion

Mitochondria are unique cell organelles representing the main protagonists of the human organism's energy supply. By creating biologically accessible energy, these organelles maintain homeostasis as well as skeletal muscle function. Therefore, accurate functioning of mitochondria is crucial to human health. Defects in the human genome affecting mitochondrial proteins can result in impaired oxidative phosphorylation capacity, the generation of ROS and further impairment of skeletal muscle structure and function. This process is thought to play an important role in human aging and specific mitochondrial and other neurodegenerative diseases such as MM and HD. The present dissertation consists of three different studies aiming to generate a detailed overview on skeletal muscle mitochondrial function and its relation to specific skeletal muscle properties.

In summary, mitochondrial fatty acid oxidative capacity is higher in advanced age and positively correlated to exercise efficiency. On the contrary, mitochondrial volume density and maximal respiratory capacity as well as skeletal muscle phenotype are not affected by aging, when fitness levels are maintained. Whereas mitochondrial quality and quantity are sustained with age, they are clearly diminished in MM. In particular, respiratory capacity of each individual complex is reduced with MM. Additionally, MM patients exhibit a shift towards more type II myofibers. These findings indicate that the disease per se may lead to reduced mitochondrial volume density and function, thereby causing the typical symptom of exercise intolerance. This in turn may potentially result in further physical inactivity and a sedentary lifestyle that aggravates diminished mitochondrial quantity and quality. Skeletal muscle (mitochondrial) properties are also affected in patients with HD. Compared to MM patients, patients with HD exhibit only slight reductions in mitochondrial respiratory capacity of complex I and maximal oxidative phosphorylation capacity. Respiratory capacities of all other complexes are not affected by this disease. In contrast to MM, HD patients demonstrate a shift from type II to more type I fibers without any alteration in mitochondrial volume density. Hence, both diseases seem to be associated with impaired aerobic energy supply, though to a varying degree, and apparently exhibit different compensatory mechanisms herefore.

Together, these studies expand previous knowledge in this field and emphasize the relevance of proper mitochondrial function for human health. Alterations in any of the respiratory complexes may have considerable implications for older individuals and for patients with MM and HD, leading to even greater risk for further functional impairments. In order to develop effective lifestyle and treatment approaches to maintain mitochondrial

function and sustain or improve quality of life, further studies on mitochondrial characteristics and underlying mechanisms are required.

4. Bibliography

The Huntington's Disease Collaborative Research Group (1993). A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. The Huntington's Disease Collaborative Research Group. *Cell* **72,** 971-983.

Alberts B, Johnson A, Lewis J, Raff M, Roberts K & Walter P (2002). The Mitochondrion. In *Mol Biol Cell*. Garland Science.

Amati F, Dube JJ, Shay C & Goodpaster BH (2008). Separate and combined effects of exercise training and weight loss on exercise efficiency and substrate oxidation. *J Appl Physiol (1985)* **105,** 825-831.

Bangsbo J, Krustrup P, Gonzalez-Alonso J & Saltin B (2001). ATP production and efficiency of human skeletal muscle during intense exercise: effect of previous exercise. *Am J Physiol Endocrinol Metab* **280,** E956-964.

Boushel R, Lundby C, Qvortrup K & Sahlin K (2014). Mitochondrial plasticity with exercise training and extreme environments. *Exerc Sport Sci Rev* **42,** 169-174.

Di Mauro S (2004). Mitochondrial medicine. *Biochim Biophys Acta* **1659,** 107-114.

Di Mauro S (2010). Pathogenesis and treatment of mitochondrial myopathies: recent advances. *Acta Myol* **29,** 333-338.

Gaesser GA & Brooks GA (1975). Muscular efficiency during steady-rate exercise: effects of speed and work rate. *J Appl Physiol (1985)* **38,** 1132-1139.

Gnaiger E (2001). Bioenergetics at low oxygen: dependence of respiration and phosphorylation on oxygen and adenosine diphosphate supply. *Respir Physiol* **128,** 277-297.

Gnaiger E (2009). Capacity of oxidative phosphorylation in human skeletal muscle: new perspectives of mitochondrial physiology. *Int J Biochem Cell Biol* **41,** 1837-1845.

Gram M, Vigelso A, Yokota T, Hansen CN, Helge JW, Hey-Mogensen M & Dela F (2014). Two weeks of one-leg immobilization decreases skeletal muscle respiratory capacity equally in young and elderly men. *Exp Gerontol* **58,** 269-278.

Hassani A, Horvath R & Chinnery PF (2010). Mitochondrial myopathies: developments in treatment. *Curr Opin Neurol* **23,** 459-465.

Hopker JG, Coleman DA, Gregson HC, Jobson SA, Von der Haar T, Wiles J & Passfield L (2013). The influence of training status, age, and muscle fiber type on cycling efficiency and endurance performance. *J Appl Physiol (1985)* **115,** 723-729.

Hoppeler H & Flück M (2003). Plasticity of skeletal muscle mitochondria: structure and function. *Med Sci Sports Exerc* **35,** 95-104.

Jacobs RA, Flück D, Bonne TC, Burgi S, Christensen PM, Toigo M & Lundby C (2013). Improvements in exercise performance with high-intensity interval training coincide with an increase in skeletal muscle mitochondrial content and function. *J Appl Physiol (1985)* **115,** 785-793.

Kirkwood SP, Munn EA & Brooks GA (1986). Mitochondrial reticulum in limb skeletal muscle. *Am J Physiol* **251,** C395-402.

Lanza IR & Nair KS (2010). Mitochondrial metabolic function assessed in vivo and in vitro. *Curr Opin Clin Nutr Metab Care* **13,** 511-517.

Larsen S, Nielsen J, Hansen CN, Nielsen LB, Wibrand F, Stride N, Schroder HD, Boushel R, Helge JW, Dela F & Hey-Mogensen M (2012). Biomarkers of mitochondrial content in skeletal muscle of healthy young human subjects. *J Physiol* **590,** 3349-3360.

Lodi R, Schapira AH, Manners D, Styles P, Wood NW, Taylor DJ & Warner TT (2000). Abnormal in vivo skeletal muscle energy metabolism in Huntington's disease and dentatorubropallidoluysian atrophy. *Ann Neurol* **48,** 72-76.

Martin JB & Gusella JF (1986). Huntington's disease. Pathogenesis and management. *N Engl J Med* **315,** 1267-1276.

Miller WL (2013). Steroid hormone synthesis in mitochondria. *Mol Cell Endocrinol* **379,** 62-73.

Mitchell P (1961). Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism. *Nature* **191,** 144-148.

Montero D & Lundby C (2015). The Effect of Exercise Training on the Energetic Cost of Cycling. *Sports Med*.

Müller W (1976). Subsarcolemmal mitochondria and capillarization of soleus muscle fibers in young rats subjected to an endurance training. A morphometric study of semithin sections. *Cell Tissue Res* **174,** 367-389.

Nelson LD & Cox MM (2008). *Lehninger Principles of Biochemistry*. Sara Tenney, W. H. Freeman and Company.

Newmeyer DD & Ferguson-Miller S (2003). Mitochondria: releasing power for life and unleashing the machineries of death. *Cell* **112,** 481-490.

Ogata T & Yamasaki Y (1997). Ultra-high-resolution scanning electron microscopy of mitochondria and sarcoplasmic reticulum arrangement in human red, white, and intermediate muscle fibers. *Anat Rec* **248,** 214-223.

Ortega JD (2013). Counterpoint: skeletal muscle mechanical efficiency does not increase with age. *J Appl Physiol (1985)* **114,** 1109-1111.

Picard M, Taivassalo T, Gouspillou G & Hepple RT (2011). Mitochondria: isolation, structure and function. *J Physiol* **589,** 4413-4421.

Picard M, White K & Turnbull DM (2013). Mitochondrial morphology, topology, and membrane interactions in skeletal muscle: a quantitative three-dimensional electron microscopy study. *J Appl Physiol (1985)* **114,** 161-171.

Pozzan T, Magalhaes P & Rizzuto R (2000). The comeback of mitochondria to calcium signalling. *Cell Calcium* **28,** 279-283.

Russ DW & Kent-Braun JA (2004). Is skeletal muscle oxidative capacity decreased in old age? *Sports Med* **34,** 221-229.

Saft C, Zange J, Andrich J, Müller K, Lindenberg K, Landwehrmeyer B, Vorgerd M, Kraus PH, Przuntek H & Schols L (2005). Mitochondrial impairment in patients and asymptomatic mutation carriers of Huntington's disease. *Mov Disord* **20,** 674-679.

Soderlund K & Hultman E (1990). ATP content in single fibres from human skeletal muscle after electrical stimulation and during recovery. *Acta Physiol Scand* **139,** 459-466.

Taanman JW (1999). The mitochondrial genome: structure, transcription, translation and replication. *Biochim Biophys Acta* **1410,** 103-123.

Taivassalo T & Haller RG (2004). Implications of exercise training in mtDNA defects--use it or lose it? *Biochim Biophys Acta* **1659,** 221-231.

Tarnopolsky MA & Raha S (2005). Mitochondrial myopathies: diagnosis, exercise intolerance, and treatment options. *Med Sci Sports Exerc* **37,** 2086-2093.

Tuppen HA, Blakely EL, Turnbull DM & Taylor RW (2010). Mitochondrial DNA mutations and human disease. *Biochim Biophys Acta* **1797,** 113-128.

Venturelli M & Richardson RS (2013). Point: skeletal muscle mechanical efficiency does increase with age. *J Appl Physiol (1985)* **114,** 1108-1109.

Walker FO (2007). Huntington's disease. *Lancet* **369,** 218-228.

Wallace DC (1999). Mitochondrial diseases in man and mouse. *Science* **283,** 1482-1488.

Westermann B (2010). Mitochondrial fusion and fission in cell life and death. *Nat Rev Mol Cell Biol* **11,** 872-884.

Wilson TM & Tanaka H (2000). Meta-analysis of the age-associated decline in maximal aerobic capacity in men: relation to training status. *Am J Physiol Heart Circ Physiol* **278,** H829-834.

Woo JS, Derleth C, Stratton JR & Levy WC (2006). The influence of age, gender, and training on exercise efficiency. *J Am Coll Cardiol* **47,** 1049-1057.

Youle RJ & van der Bliek AM (2012). Mitochondrial fission, fusion, and stress. *Science* **337,** 1062-1065.

Zeviani M & Di Donato S (2004). Mitochondrial disorders. *Brain* **127,** 2153-2172.

5. Curriculum Vitae

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Education

2003 – 2007	Kantonsschule Ausserschwyz, Nuolen, Switzerland *Main subject: Mathematics and Physics*
2007 – 2010	ETH Zurich, Zurich, Switzerland *Bachelor of Science ETH in Human Movement Sciences*
2010 – 2012	ETH Zurich, Zurich, Switzerland *Master of Science ETH in Human Movement Sciences* *Major in Exercise Physiology* *Master Thesis: "Multiday acute sodium-bicarbonate intake improves endurance capacity and reduces acidosis in men"*
2009 – 2013	ETH Zurich, Zurich, Switzerland *Master of Advanced Studies, Teaching diploma in Sports*
2013 – present	Zurich Center for Integrative Human Physiology (ZIHP), University and University Hospital Zurich, Zurich, Switzerland *Employment as PhD student since 01.01.2013* *PhD Thesis: "Skeletal Muscle Mitochondrial Function in Human Health and Disease"*

Work Experience

2012 – 2015	ETH Zurich, Department of Health Sciences and Technology, Zurich, Switzerland *Assistant Assessment II, Athletics I + II*

Presentations and grants

- Multiday acute sodium-bicarbonate intake improves endurance capacity and reduces acidosis in men
  Poster Presentation, ZIHP-Symposium, August 23, 2013, Zurich, Switzerland
- Exercise efficiency increases with age but is not related to mitochondrial function
  Oral Presentation, ZIHP-Symposium, August 29, 2014, Zurich, Switzerland
- Exercise efficiency is higher in old compared to young men at similar fitness level
  Oral Presentation, ECSS 2015, June 25, 2015, Malmö, Sweden
- Project Grant, 2014, *Neuromuscular Research Association Basel, Basel, Switzerland*
- Travel Grant, 2015, *Hartmann-Müller Stiftung, Zurich, Switzerland*
- Travel Grant, 2015, *Swiss Physiological Society, Zurich, Switzerland*

Publications

- Jacobs RA, Lundby AM, Fenk S, *Gehrig S*, Siebenmann C, Flück D, Kirk N, Hilty MP, Lundby C. Twenty-eight days of exposure to 3,454 m increases mitochondrial volume density in human skeletal muscle. J Physiol. 2015 Sep 4.